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14. ABSTRACT

During the funding period, the PI has found that disrupting *Brca1* by either germline or epithelium-specific mutation in p18-deficient mice activates epithelial-to-mesenchymal transition (EMT) and induces dedifferentiation of luminal stem cells (LSCs), which associate closely with expansion of basal and cancer stem cells and formation of basal-like tumors. Mechanistically, BRCA1 bound to the *TWIST* promoter, suppressing its activity and inhibiting EMT in mammary tumor cells. In human luminal cancer cells, BRCA1 silencing was sufficient to activate TWIST and EMT and increase tumor formation. In parallel, *TWIST* expression and EMT features correlated inversely with *BRCA1* expression in human breast cancers. Together, our findings showed that BRCA1 suppressed TWIST and EMT, inhibited LSC dedifferentiation and repressed expansion of basal stem cells and basal-like tumors. Thus, our work offers the first genetic evidence that Brca1 directly suppresses EMT and LSC de-differentiation during breast tumorigenesis.

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1. Introduction

Mammary epithelia are mainly composed of luminal and basal cells whose expansion and maintenance in adult mice are ensured by luminal and basal stem cells, respectively(1-2). Accordingly breast cancer is divided into two major subtypes—luminal and basal-like tumors—that develop through distinct mechanisms and exhibit different responsiveness to treatment(3). Mutation of *BRCA1* is frequently associated with basal-like breast cancer(4-7). How Brca1 controls cell lineage commitment, maturation and transformation in the mammary gland and tumor development remain to be defined and are the focus of this application.

p18^{Ink4c} (*p18*), an inhibitor of CDK4/6 and activator of the RB pathway, expresses significantly lower in human breast cancers, and deletion of *p18* in mice stimulates mammary luminal stem cell proliferation and leads to luminal tumor development(8). *Brca1* mutation in mice causes premature senescence making it very difficult to determine the mechanism of *Brca1* in the suppression of mammary tumors. Taking advantage of p18 deficient mice that rescue the growth defects caused by mutation of *Brca1*(9), we are able to study the role of *Brca1* in controlling mammary cell fate and tumor development. We discovered that mutation of *Brca1* altered luminal cell fate, down-regulated the expression of luminal differentiation genes, up-regulated the expression of basal genes, and activated Twist and other epithelial-mesenchymal transition (EMT)-inducing transcription factors in p18 deficient luminal and tumor cells. Germline mutation of *Brca1* converts p18 deficient luminal type tumors into basal-like tumors with EMT features. Ectopic expression of WT BRCA1 in *BRCA1* mutant human basal-like cancer cells suppresses Twist and EMT and knockdown of BRCA1 sensitizes luminal cancer cells to induction of Twist and EMT in response to TGFβ.

Based on these findings, we hypothesize that Brca1 suppresses Twist and EMT to prevent luminal stem and tumor cells from aberrant basal and mesenchymal differentiation. Reduction or loss of Brca1 activates Twist and EMT, which allow LSCs to gain a multipotent capacity and cancer stem cells (CSC) to enhance self-renewal potential and lead to luminal-to-basal and luminal-to-mesenchymal cell transformation. We propose three specific aims to test this hypothesis: (1) to determine the role of BRCA1 in suppressing EMT and basal differentiation of mammary luminal cells, (2) to determine the function of BRCA1 in suppressing EMT of breast cancer stem cells, and (3) to determine the molecular mechanism of Brca1 in suppressing TWIST.

2. Keywords

BRCA1, *p18^{ink4c}*, EMT, Luminal stem cell, Basal-like tumor

3. Overall Project summary

(1) Germline mutation of *Brca1* transforms *p18$^{-/-}$* luminal tumors into basal-like tumors with induction of EMT

In our previous studies, we reported that deletion of p18 in mice stimulates mammary LSC proliferation and leads to spontaneous luminal tumor development(8), and that germline mutation of *Brca1* in p18-deficient mice with Balb/c enriched background blocks the expansion of LSCs and transforms luminal tumors into basal-like tumors(9). Prompted by the highly invasive heterogeneous mammary tumors developed in *p18$^{-/-}$;Brca1$^{+/-}$* mice with various degrees of whorls and clusters of spindle-shaped cells within these tumors – typical morphological characteristics of mesenchymal cells(9) – we looked at molecular markers associated with EMT. We found that the majority of the luminal tumors from *p18$^{-/-}$* mice highly expressed E-cadherin (Cdh1), an epithelial marker, whereas basal-like tumors from *p18$^{-/-}$;Brca1$^{+/-}$* mice expressed very weak and heterogeneous Cdh1. In contrast, most (77%, n=13) of *p18$^{-/-}$;Brca1$^{+/-}$* tumors that developed after one year of age were stained positive for mesenchymal markers including fibronectin (Fn), vimentin (Vim), and CD29, while only 11% (n=19) of *p18$^{-/-}$* tumors that developed at a similar age were positive for these markers (Fig. 1A-C, Table 1). This observation suggests that heterozygous germline mutation of *Brca1* activates EMT in mammary tumor progression.

Consistently, *p18$^{-/-}$;Brca1$^{+/-}$* tumor cells that were positive for Ck5 expressed very low levels of Cdh1 and the majority of Fn positive cells co-expressed Ck5 (Fig. 1B). These data suggest, at the least, that some Ck5+ basal-like tumor cells lost their epithelial characteristics and gained mesenchymal features. In further analysis of these tumors for the expression of CD29 , a basal and mesenchymal marker(10) demonstrated to be enriched in breast CSCs(11-12), we found that 69% (n=13) of *p18$^{-/-}$;Brca1$^{+/-}$* tumors expressed various degrees

of CD29 positive tumor cells from 2%-60% while only 11% (n=19) of *p18*$^{-/-}$ tumors were positive for CD29 in 2-3% of tumor cells (Fig. 1C, Table 1). These observations support the notion that EMT activation, as previously demonstrated(10, 13), results in cancer cells gaining stem cell properties. Primary *p18*$^{-/-}$;*Brca1*$^{+/-}$ tumor cells formed more and larger colonies in matrigel than *p18*$^{-/-}$ tumor cells (Fig. 1E) and Ck5/Ck8 double positive tumor cells were frequently detected in *p18*$^{-/-}$;*Brca1*$^{+/-}$ tumors but rarely in *p18*$^{-/-}$ tumors, 1.1% (67/6100) vs. 0.04% (2/5120) (Fig. 1F and (8-9)) which further suggests increased CSCs in *p18*$^{-/-}$;*Brca1*$^{+/-}$ tumors. Together, these results indicate that heterozygous germline mutation of *Brca1* induces EMT, increases CSCs, and transforms p18 null luminal tumors into basal-like tumors.

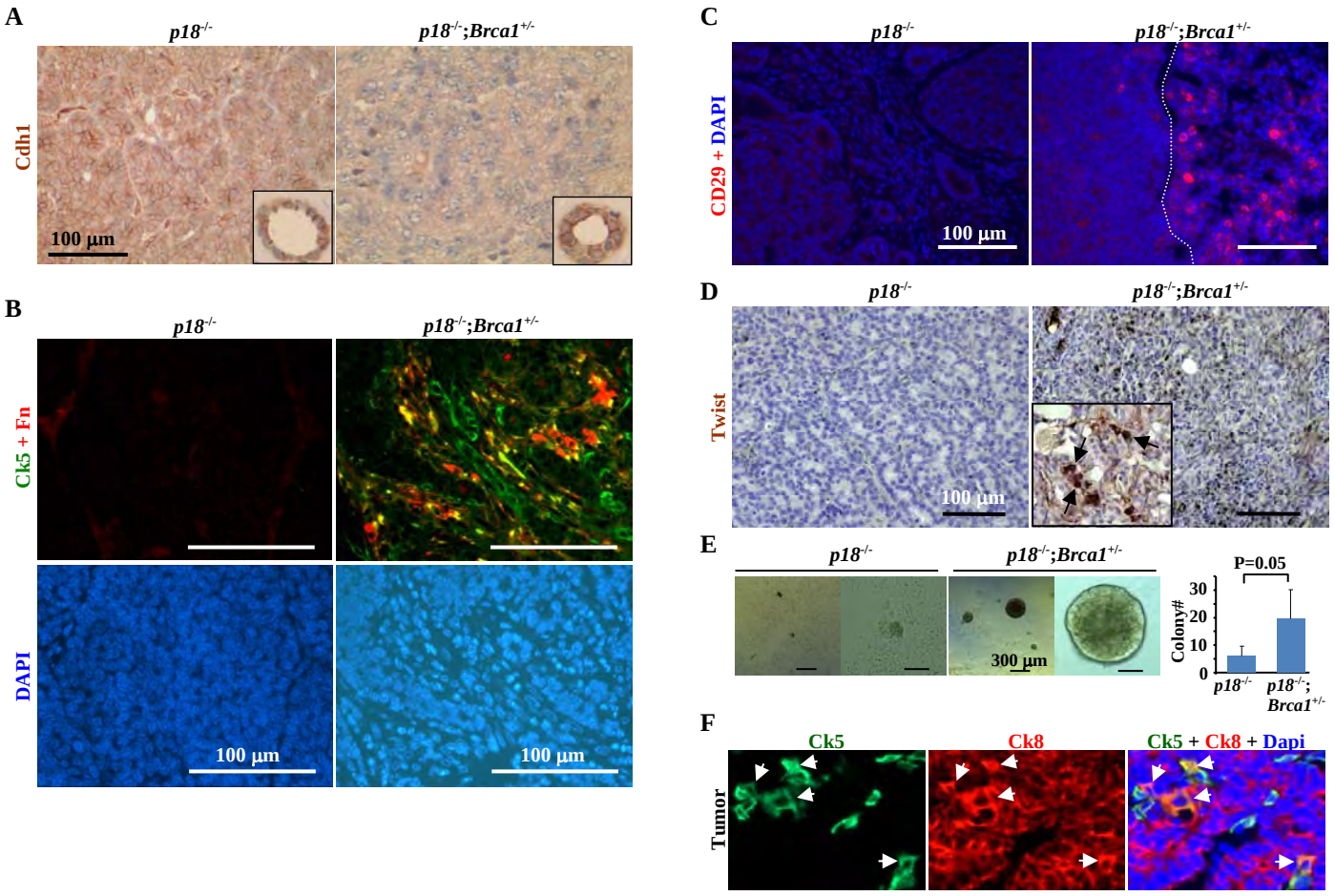


Figure 1. *Brca1* heterozygosity transforms p18-deficient luminal tumors into basal-like tumors with EMT features. (A-D) Representative immunostaining of tumors with Cdh1 (A), Ck5 and Fn1 (B), CD29 (C), and Twist (D). The inset in (A) shows staining of normal glands in the same mouse and in (D) shows staining of lung metastasis. (E) Tumor cells were cultured for two weeks and colonies larger than 30 m were counted. The bar graphs represent the mean ± SD of two tumors per genotype.
(F) Representative immunostaining of tumors from *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice with Ck5 and Ck8. Ck5+Ck8+ cells are indicated.

(2) Germline mutation of *Brca1* activates EMT-TFs in mammary and tumor development

We then determined the expression of EMT-TFs and observed that 77% (n=13, >one year of age) of *p18*$^{-/-}$;*Brca1*$^{+/-}$ tumors were stained positive for Twist, Foxc1, Foxc2, Slug, and Snail in greater than 2% of cells per tumor whereas 16% (n=19, >one year of age) of *p18*$^{-/-}$ tumors were positive at similar ages (Fig. 1D, Table 1). Tumors with high expression of EMT-TFs showed high histological grade and strong invasive and metastatic potential as evidenced by EMT-TF positive staining in the invasive front of tumors and metastasized cancers (Fig. 1D). The expression pattern and percentage of positive cells in tumors stained for EMT-TFs and EMT markers were highly correlated with its genotype – *p18*$^{-/-}$ or *p18*$^{-/-}$;*Brca1*$^{+/-}$, – which not only confirms that germline mutation of *Brca1* promotes EMT in mammary tumors but that this induction of EMT is very likely a result of the aberrant activation of EMT-TFs in *Brca1* mutant tumors. We next isolated mammary epithelial cells (MECs) from tumor-free virgin mice and found that *Brca1*$^{+/-}$ and *p18*$^{-/-}$;*Brca1*$^{+/-}$ cells expressed

significantly less *Cdh1* and more EMT-TFs than WT or *p18*$^{-/-}$ cells (data not shown). These results indicate that EMT-TF activation in *Brca1* mutant MECs occurs prior to tumor initiation.

Tumor	**Wt**		***p18*$^{-/-}$**		***Brca1*$^{+/-}$**		***p18*$^{-/-}$*;Brca1*$^{+/-}$**	
	<12 m	**12-27 m**	**<12 m**	**12-22 m**	**<12 m**	**12-27 m**	**<12 m**	**12-22 m**
Mammary Tumor	0/5	1/10[a] (10%)	4/16 (25%)	19/23[b] (83%)	0/3	1/11[c] (9%)	6/16 (38%)	13/15[d] (87%)
Metastasis[e]		0/1	0	1/19		0/1	0	4/13
ERα+ tumor **% ERα+ cells/tumor**		1/1 5%	3/4 2-40%	15/19 2-40%		0/1	1/6 <2%	2/13 <2%
Ck5+ tumor **%Ck5+ cells/tumor**		0/1	0/4	3/19 [f] 1-5%		1/1 ~2%	4/6 2-20%	11/13[g] 2-95%
EMT marker+ tumor[h]		0/1	0/4	2/19 (11%)		0/1	2/6 (33%)	10/13 (77%)
EMT-TF+ tumor[i]		0/1	0/4	3/19 (16%)		1/1 (100%)	3/6 (50%)	10/13 (77%)

Table 1. Characterization of spontaneous mammary tumors derived from mutant mice in Balb/c enriched background
[a] 24-month-old tumor-bearing mouse.
[b] Most tumor-bearing mice were 12-16 months old and the oldest was 22 months old. One male developed mammary tumor.
[c] 25.5-month-old tumor-bearing mouse.
[d] Most tumor-bearing mice were 12-16 months old, and the oldest was 20 months old. One male developed mammary tumor.
[e] Mammary tumors metastasized mostly to the lung except one to a blood vessel in a *p18*$^{-/-}$;*Brca1*$^{+/-}$ mouse.
[f] One tumor stained positive for Ck5 in ~5% tumor cells and the other two were positive in ~1% tumor cells.
[g] Two tumors stained positive for Ck5 in ~95% tumor cells.
[h] At least two EMT markers (decreased Cdh1, increased Vim, Fn1, Sma or Cd29) were detected in >2% tumor cells.
[i] At least two EMT-inducing transcription factors (EMT-TFs), which include Twist, Slug, Snail, Foxc1 and Foxc2, stained positive in >2% tumor cells.

(3) Specific deletion of *Brca1* in mammary epithelia activates EMT and induces aberrant differentiation of LSCs

To directly test the function of Brca1 in controlling and transforming MECs as well as to determine the implications of loss of Brca1 on mammary tumorigenesis, we generated *Brca1*$^{f/f}$;MMTV-cre^{+} and *Brca1*$^{f/-}$;MMTV-cre^{+} mice with and without *p18* mutation in Balb/c-B6 mixed background, in which MMTV-cre (MC) is active in virgin epithelia but not in stroma(14-15). Using these mice also enabled us to rule out the impact of *Brca1* mutant stroma on mammary stem cell self-renewal and tumorigenesis.

Brca1$^{f/-}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC breasts expressed <5% of Brca1 protein and mRNA relative to the levels in *Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC, indicating an efficient and near complete depletion of Brca1 in the mammary epithelia (Fig. 2A, B). Similarly, *Brca1*$^{f/f}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC breasts expressed <20% of Brca1 protein and mRNA relative to the levels in MC and *p18*$^{-/-}$;MC (data not shown). Consistent with the data from *Brca*$^{+/-}$ mice(9), the expression of *Gata3*, *Cdh1*, and *Epcam* – genes associated with luminal differentiation – in *Brca1*$^{f/-}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC breasts was significantly reduced relative to *Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC breasts (Fig. 2A, B), suggesting that loss of Brca1 impairs luminal differentiation. MECs from *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice showed increased mammosphere-forming ability than those from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mice. Most *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mammospheres were 35-45μm and none larger than 100μm whereas 10-15% of *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mammospheres were larger than 100μm. The average *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mammosphere was significantly larger than that of *p18*$^{-/-}$;*Br*$^{f/+}$;MC mammospheres (Fig. 2C). These results suggest that Brca1 deficiency increased the self-renewal capacity of *p18*$^{-/-}$ mammary stem cells. Accordingly, MECs from *p18*$^{-/-}$;*Brca1*$^{f/-}$;*MC* mice formed more colonies than those from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mice and *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mammospheres expressed significantly higher levels of EMT-TFs than those of *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC (Fig. 2D, E). These results confirms that loss of Brca1 activates EMT-TFs, which is likely responsible for the induction of EMT and increased mammosphere- and colony-forming potential in *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC MECs.

We then performed FACS and found that *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC MECs had a reduced CD24^{+}CD29^{-} LSC-enriched population and increased CD24^{+}CD29^{+} BSC-enriched population compared to *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC MECs at 22 weeks of age (Fig. 2F). Similar, but less significant, trends were also observed in *p18*$^{-/-}$

;*Brca1*$^{f/+}$;MC mice relative to *p18*$^{-/-}$;MC mice at 16 weeks of age (data not shown). These results suggest that Brca1 deficiency results in the expansion of BSCs and blockage of LSCs, the latter of which is consistent with our findings derived from heterozygous germline *Brca1* mutant mice(9).

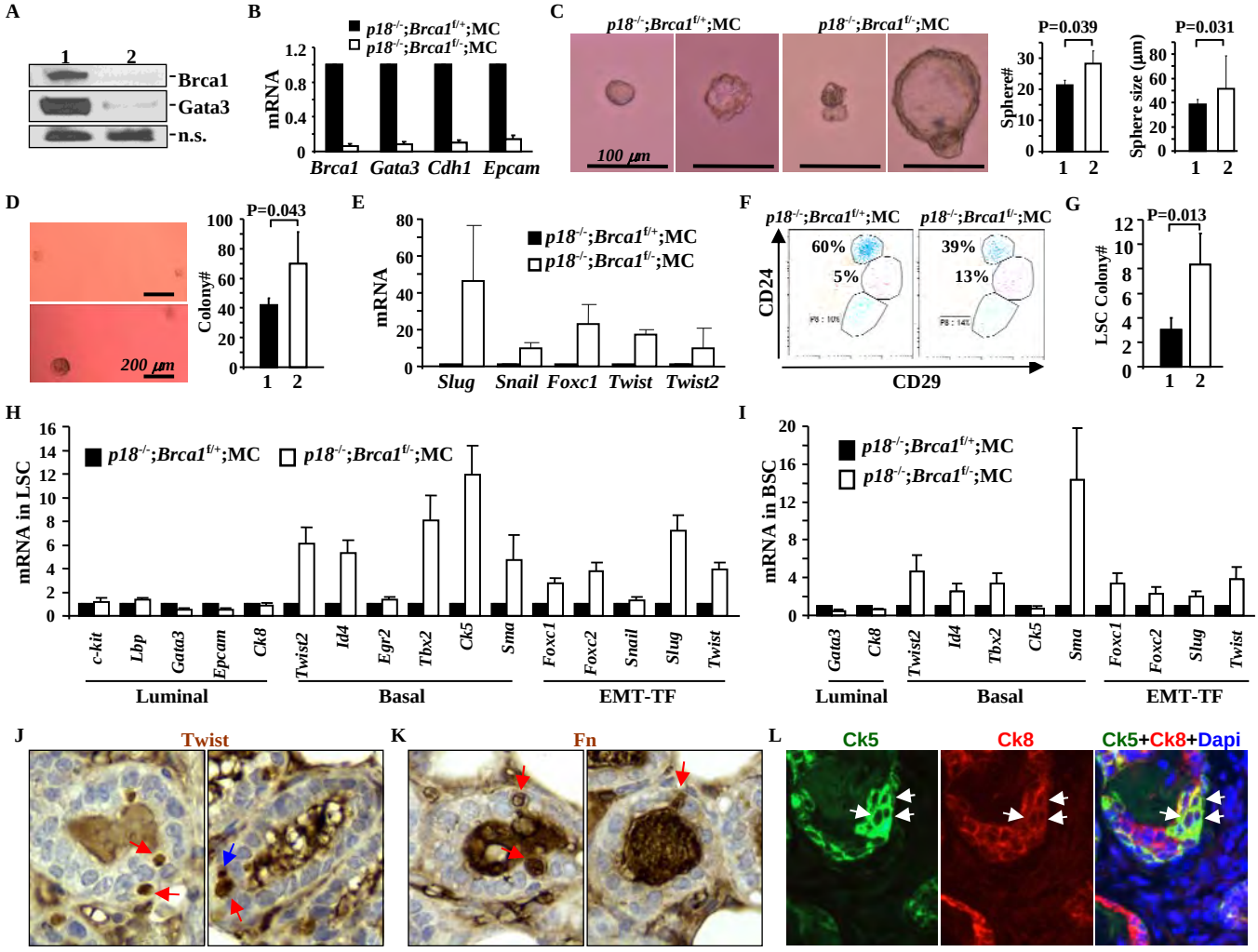


Figure 2. Deletion of Brca1 in mammary epithelia inhibits luminal differentiation and activates EMT-TFs in mammary stem cells. (A, B) Mammary tissues from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC (Lane 1) and *p18*$^{-/-}$;*Brca*$^{f/-}$;MC (Lane 2) mice were analyzed by western blot (A), and Q-RT-PCR (B). n.s., non-specific band. Q-RT-PCR data are expressed as the mean ± SD from triplicates of each of three separate mice. (C, D) Mammary cells were analyzed by mammosphere (C) and colony formation assay (D). The number of spheres larger than 30μm, the sizes of spheres, and the number of colonies larger than 30 m were quantified.1, *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC; 2, *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC. The bar graphs represent the mean ± SD of two animals per genotype. (E) RNA from mammospheres was analyzed. Data are expressed as mean ± SD from triplicates of each of two separate mice. (F) Mammary cells were analyzed by flow cytometry. (G) FACS-sorted LSCs from (F) were analyzed by colony formation assay. The bar graphs represent the mean ± SD of two animals per genotype. 1, *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC; 2, *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC. (H, I) RNA from LSCs (H) and BSCs (I) was analyzed. Data are expressed as the mean ± SD from triplicates of each of two separate mice. (J, K, L) Tumor-free mammary glands from *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice were stained with antibodies against Twist (J), Fn (K), Ck5 and Ck8 (L). Twist or Fn positive ULLC (red arrows) and SLC (blue arrows), as well as Ck5+Ck8+ epithelial cells (white arrows) are indicated.

FACS-sorted cells of the BSC-enriched population expressed higher basal genes (*Twist2*, *Id4*, and *Tbx2*) and lower luminal genes (*c-kit*, *Epcam*, and *Gata3*) than those of the LSC-enriched population, confirming that these cell populations are, as reported(16), the basal and luminal cell enriched populations, respectively (data not shown). LSCs derived from *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice formed more colonies in matrigel and expressed lower luminal and epithelial genes and significantly higher basal genes and EMT-TFs when compared with *p18*;*Brca1*$^{f/+}$;MC LSCs (Fig. 2G, H). Consistently, LSCs from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mice also expressed lower luminal genes and higher basal genes and EMT-TFs than those from *p18*$^{-/-}$;MC mice (data not shown). These

results indicate that haploid or near complete loss of *Brca1* in mammary epithelium not only inhibits the expression of luminal genes but also stimulates the expression of basal genes and EMT-TFs in *p18*$^{-/-}$ LSCs. Interestingly, expression of basal genes and EMT-TFs was also significantly increased in the BSCs from *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice relative to those from *p18*;*Brca1*$^{f/+}$;MC mice (Fig. 2I). Together, these results suggest that Brca1 deficiency results in the expansion of BSCs that is likely, at least partially, resulting from the dedifferentiation of LSCs.

We have previously analyzed five histologically distinct epithelial cell populations and defined the small light cell (SLC) and undifferentiated large light cell (ULLC) populations as enriched for stem and luminal stem/progenitor cells, respectively(8). To determine the impact of EMT on stem/progenitor cell populations *in situ*, we examined tumor-free mammary glands and found that Twist or Fn positive MECs were frequently detected in *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC or *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice but not in *p18*$^{-/-}$;MC mice and that most, if not all, Twist or Fn positive cells were either SLC or ULLC, ULLC in particular (Fig. 2J, K). Furthermore, Ck5 and Ck8 double positive epithelial cells were also frequently detected in *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC but not in *p18*$^{-/-}$;MC mammary (Fig. 2L, and data not shown). These results further suggest that loss of Brca1 in MECs activates Twist, induces EMT, and leads to dedifferentiation of LSCs.

(4) Specific deletion of Brca1 in mammary epithelia recapitulates basal-like tumorigenesis and EMT activation

To determine the tumorigenic impact of specific loss of Brca1 in mammary epithelia, we first examined *Brca1*$^{f/-}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice and found that no hyperplasia nor tumors developed in 5 female *Brca1*$^{f/-}$;MC mice at 10-12 months of age. Of the 8 *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice examined at similar ages, all developed mammary hyperplasia though no mammary tumors were detected. A majority (7/8) of *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice died at early ages from carcinomas in the pancreas, skin, pituitary or lung (data not shown), very likely due to active MMTV-Cre expression and near complete deletion of Brca1 in these tissues(17), which prevented us from observing the relatively late onset mammary tumorigenesis in these mice. These results, however, confirm the previous findings that loss of Brca1 alone is insufficient to promote tumorigenesis and that Brca1 cooperates with p18 to control tumorigenesis.

We then examined *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice and found that 1 of 4 *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mice and 4 of 5 *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice developed mammary tumors in 12-16 months (Fig. 3). In accordance with the tumors developed in *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice, mammary tumors in *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice were also highly heterogenous, poorly differentiated, and more aggressive than those developed in *p18*$^{-/-}$ mice (Fig. 3, Fig. 1, and data not shown). About 25-30% *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC tumor cells were spindle-shaped and were positive for Twist and Fn (Fig. 3A, B), and more than 40% of the tumor cells were positive for Ck5 and negative for Cdh1 or Ck8 (Fig. 3C, D), indications of a basal-like tumor undergoing EMT. The *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mammary tumors also expressed 1/3 of *Brca1* and 1/5 of *Gata3* relative to the tumor-free mammary tissues of the same mouse (Fig. 3E), confirming deficient Brca1 and downregulation of Gata3 in the tumor.

More than 25% of tumor cells were spindle-shaped in all four *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mammary tumors and two displayed more than 90% spindle-shaped cells (Fig. 3F). These tumors were also positive for Twist and Fn (Fig. 3G), indications of typical metaplastic breast carcinomas undergoing EMT. A *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC tumor expressed less than 10% *Brca1* and *Gata3* when compared to tumor-free mammary of the same mouse (Fig. 3I). FACS showed that the LSC-enriched population in *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mammary tumors was significantly reduced in comparison to the tumor-free mammary tissues of the same mouse (6% versus 56%) and when compared to *p18*$^{-/-}$ mammary tumor cells (6% versus 57%). Contrastingly, the BSC-enriched population, also enriched with breast CSCs, was significantly expanded in *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mammary tumors relative to the tumor-free mammary tissues of the same mouse (19% versus 11%) and when compared to *p18*$^{-/-}$ mammary tumor cells (19% versus 4%) (Fig. 3H). These results further support that *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mammary tumors are basal-like tumors undergoing EMT that are enriched with CSCs, which is in line with the data derived from human patients showing that metaplastic breast carcinomas are basal-like breast cancers with EMT-like molecular make-up and are closely correlated with BRCA1 dysfunction(18).

Taken together, these results suggest that insufficient Brca1 in mammary epithelial cells represses Gata3, activates Twist and EMT, and results in basal-like tumorigenesis with an increase in the CSC population. Since *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice are in B6 and Balb/c mixed backgrounds, unlike *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice in pure Balb/c background, these data also suggest that the role of Brca1 controlling basal-like tumorigenesis and EMT is independent of genetic background.

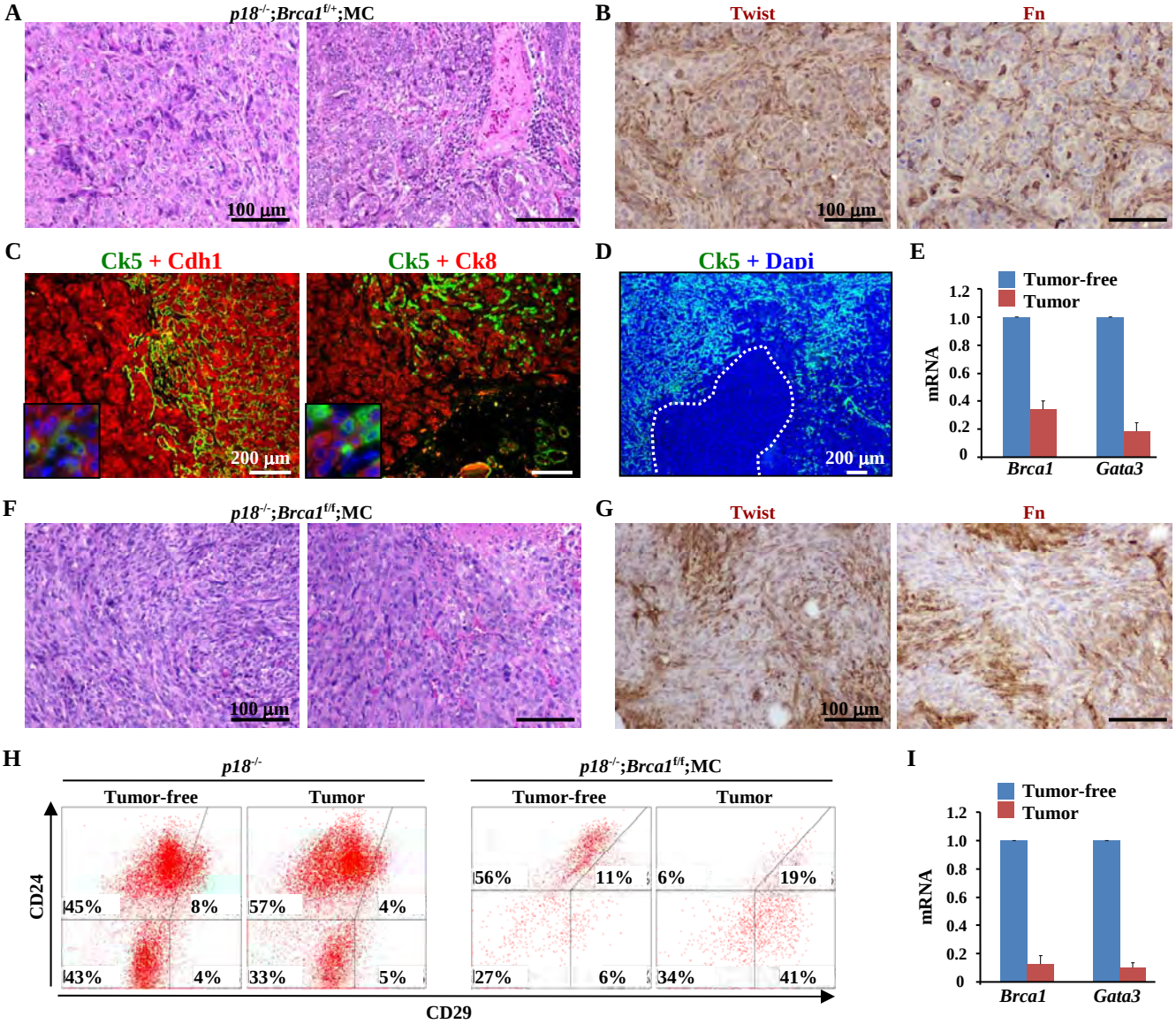


Figure 3. Deletion of Brca1 in mammary epithelia recapitulates basal-like tumor formation and EMT activation. Mammary tumors derived from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC (A-E) or *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC (F-I) mice were stained with H.E (A, F), Twist or Fn (B, G), Ck5 and Cdh1 or Ck8 (C), Ck5 (D), or analyzed by Q-RT-PCR (E, I) and FACS (H). Tumor-free mammary cells or tissues from the same mouse were used as controls. Q-RT-PCR data are expressed as the mean ± SD from triplicates.

We transplanted primary tumor cells derived from *p18*$^{-/-}$ and *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice into mammary fat pad (MFP) of NSG mice. We found that *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC tumor cells regenerated basal-like breast tumors in recipient mice whereas *p18*$^{-/-}$ cells did not yield tumors in the same time period with the same cell numbers (Fig. 3B, C). These results suggest that loss of Brca1 in p18 deficient mice enhances CSC properties.

Genotype	# of tumor cells transplanted	Tumor incidence*
p18$^{-/-}$	3 x 10^6	0/4
p18$^{-/-}$;Brca1$^{F/F}$;MC	3 x 10^6	3/3
	0.5 x 10^6	2/2
	0.1 x 10^6	4/4

Table 2. Loss of Brca1 promotes CSC properties. Primary tumor cells were transplanted into MFP of NSG mice. 4 weeks post-transplantation, tumors developed in recipient mice were counted and analyzed. All regenerated tumors are basal-like tumors and 0.8-1.3 cm^3 in size

(5) BRCA1 suppresses TWIST transcription and EMT

We screened a panel of human breast cancer cell lines and found that MCF7 and T47D cells expressed higher CDH1 and GATA3 and lower VIM and EMT-TFs than SUM149 and HCC1937 cells (data not shown), confirming that MCF7 and T47D cells are luminal/epithelial-like and SUM149 and HCC1937 cells are basal/mesenchymal-like cancer cells in our culture system(19). Transfection of WT *BRCA1* into HCC1937 (*BRCA1* mutant, transcriptionally null) cells resulted in increase of *CDH1* and decrease of *VIM* and *FN*, indicating that BRCA1 suppresses EMT. Importantly, ectopic expression of BRCA1 significantly repressed *TWIST* by more than 50% compared to control, moderately repressed *FOXC2*, but hardly repressed other EMT-TFs (Fig. 4A). A similar inhibitory effect on *TWIST* and *FOXC2* expression was also detected in 293T cells transfected with BRCA1 (data not shown). Since the ability of BRCA1 in regulating transcription controls normal differentiation and suppresses tumor development(20-21), we determined whether BRCA1 is recruited to the *TWIST* promoter. A previous study demonstrated that GATA3 recruits BRCA1 to its binding sites in the *FOXC1/2* promoters to repress their transcription(22). We performed bioinformatic analysis of the *TWIST* gene promoter and found that there exists, at the least, six putative GATA3 binding sites on the *TWIST* promoter (Fig. 4B), which are conserved in both human and mouse (data not shown). We then performed a ChIP assay and found that one of five amplicons that contained two GATA3 sites was specifically enriched in the immunoprecipitation of BRCA1 in HCC1937 cells transfected with WT *BRCA1* compared to control (P5 in Fig. 4C). In sum, these results suggest that BRCA1 specifically binds to the TWIST promoter and negatively regulates its transcription.

To confirm the role of Brca1 in the suppression of Twist and tumor development *in situ*, primary mammary tumors derived from *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice were immunostained with antibodies against Brca1 and Twist. We found that tumor cells positive for Brca1 expressed very low or no Twist whereas Brca1 mutant tumor cells expressed high levels of Twist, most of which were spindle-shaped basal-like cells (Fig. 4D), demonstrating that Brca1 inhibits Twist and EMT in mammary tumor development and progression.

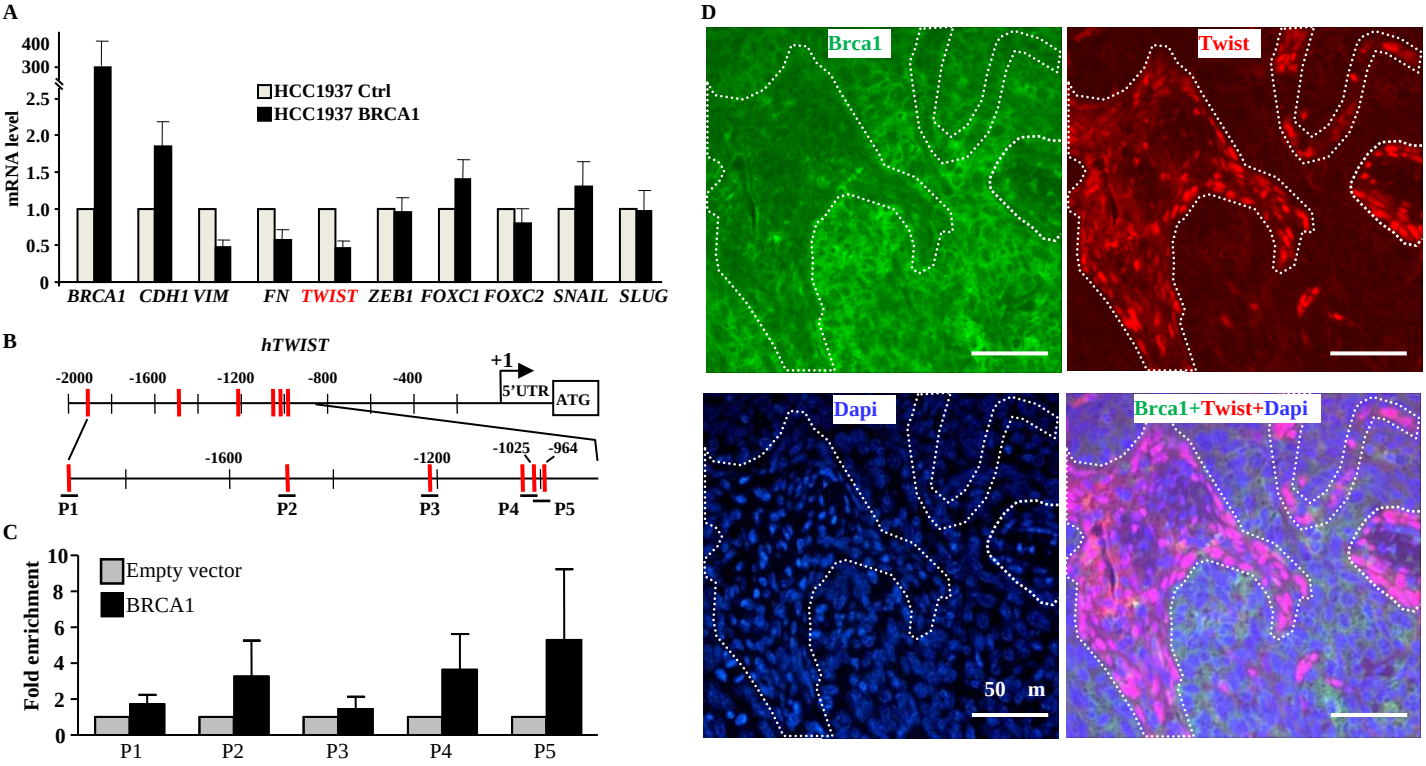


Figure 4. BRCA1 suppresses TWIST and EMT in mammary tumor cells. (A) HCC1937 cells were transfected with pcDNA3-empty (Ctrl) or pcDNA3-BRCA1 (BRCA1) and RNA was analyzed. Data are expressed as the mean ± SD from triplicates of two independent experiments. (B) Diagram showing the locations of putative GATA3 sites in the human *TWIST* gene. (C) ChIP analysis of BRCA1 binding to putative GATA3 sites on the *TWIST* promoter in HCC1937 cells transfected with *BRCA1*. Data are expressed as the mean ± SD from triplicates of two independent experiments. (D) Mammary tumors from *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice were stained with antibodies against Brca1 (green) and Twist (red).

(6) Knockdown of BRCA1 in activates TWIST and EMT with enhanced tumor formation potential

We knocked down BRCA1 in two human luminal cancer cell lines, MCF7 and T47D, using BRCA1 shRNA targeting 3 different sequences (Fig. 5A, and data not shown), and transplanted these cells into the mammary fat pads of NSG mice. We found that mammary tumors from T47D-Sh-BRCA1 cells were palpable in eight weeks whereas tumors formed from T47D-Sh-Ctrl. cells were undetectable at this stage (data not shown). Eighteen weeks after transplantation, T47D-Sh-BRCA1 tumors were significantly bigger than T47D-Sh-Ctrl. tumors (Fig. 5B, C). Consistently, mammary tumors from MCF7-Sh-BRCA1 cells were palpable significantly sooner and were larger compared to tumors from MCF7-Sh-Ctrl. cells (data not shown). These results indicate that knockdown (KD) of BRCA1 in luminal cancer cells enhances their tumor formation potential. Histo- and pathological analysis revealed that, unlike homogeneous and well differentiated T47D-Sh-Ctrl. mammary tumors, T47D-Sh-BRCA1 tumors were highly heterogenous with an abundance of large and poorly differentiated cells (Fig. 5D), suggesting that KD of BRCA1 induced the dedifferentiation of luminal tumor cells. IHC analysis indicated that most cells in T47D-Sh-BRCA1 tumors expressed very faint or no CDH1 and ER but high levels of TWIST, VIM, and FN in comparison to T47D-Sh-Ctrl. tumors (Fig. 5E-G, data not shown), indicating that T47D-Sh-BRCA1 tumors cells had undergone EMT. These results collectively suggest that KD of BRCA1 in breast luminal tumor cells activates TWIST and EMT which is associated with increased tumor formation potential, further supporting the data derived from *p18*;*Brca1* double mutant mice.

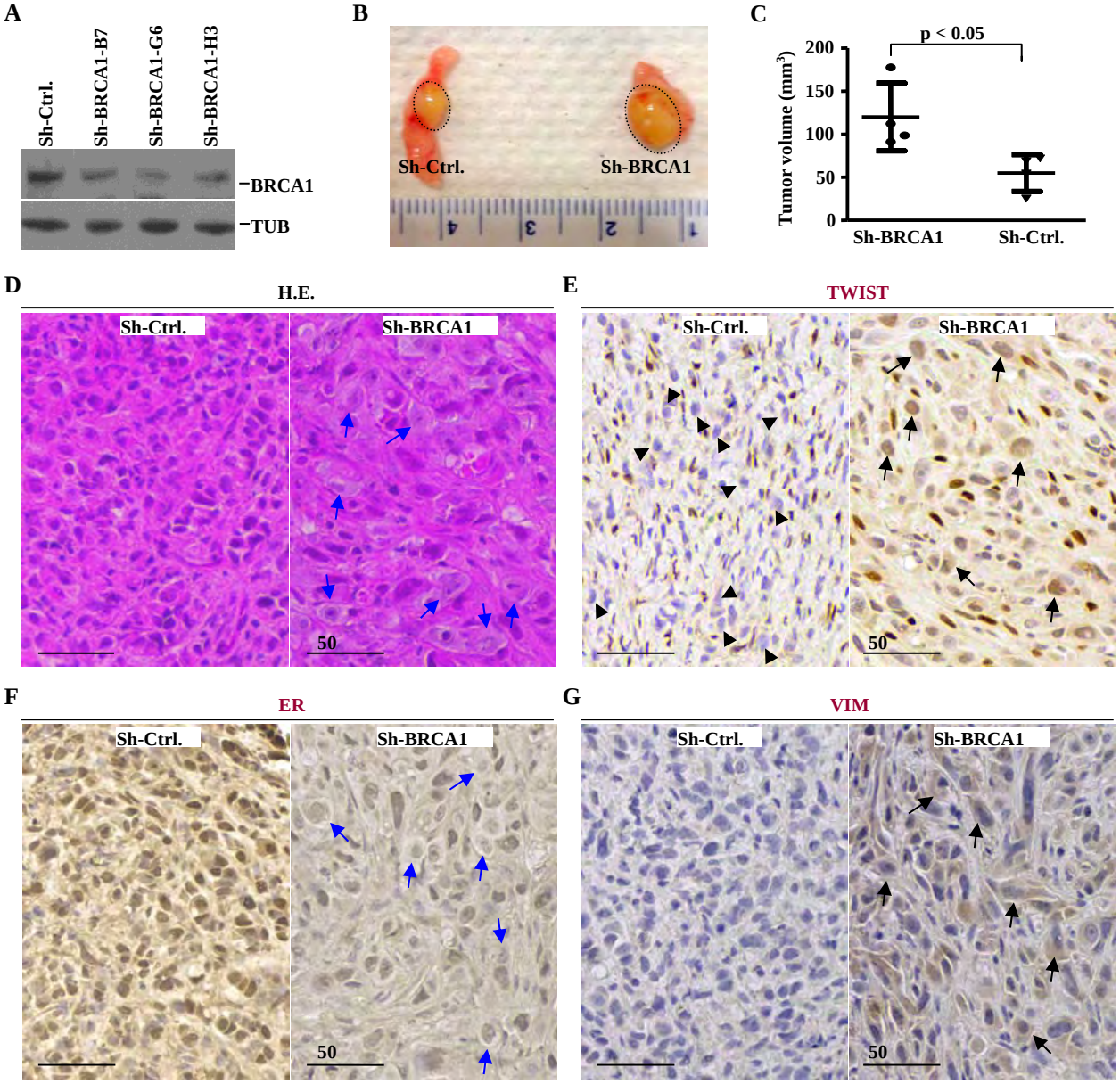
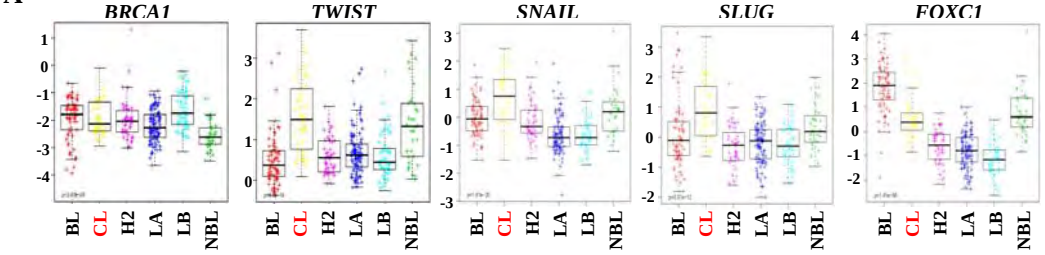


Figure 5. Knockdown of BRCA1 in luminal cancer cells increases tumor formation potential and TWIST expression
(A) T47D cells were infected with either pGIPZ-empty (sh-Ctrl.) or pGIPZ-shBRCA1 targeting different sequences of human *BRCA1* (sh-BRCA1-B7, sh-BRCA1-G6, and sh-BRCA1-H3). Cells stably expressing sh-Ctrl or shBRCA1 were analyzed by Western blot. (B, C) T47D cells stably expressing sh-Ctrl or shBRCA1-G6 (sh-BRCA1) were transplanted into the mammary fat pads of female NSG. Representative gross appearance of tumors formed is shown (B) and tumor volumes were plotted (C). Values represent the average tumor volumes ± SD of 4 tumors. (D - G) Mammary tumors formed by transplantation of T47D cells stably expressing sh-Ctrl or sh-BRCA1 were stained with H.E (D), TWIST (E), ER (F), and VIM (G). Note the highly heterogeneous Sh-BRCA1 tumor cells that are positive for TWIST and VIM (black arrows) and negative for TWIST (black arrowheads) and for VIM in Sh-Ctrl. tumors. Large and less-differentiated cells in Sh-BRCA1 tumors are indicated by blue arrows.

(7) BRCA1 and *TWIST* expression are inversely related in human claudin-low type breast cancers

Gene expression profiling analyses have categorized human breast tumors into six intrinsic subtypes: basal-like (BL), claudin-low (CL), Her2+ (H2), luminal A (LA), luminal B (LB), and normal breast-like (NBL), each of which has unique biological and prognostic features(23). Of these subtypes of breast cancer, the CL subtype is characterized by the low to absent expression of luminal differentiation markers and high enrichment for EMT markers and cancer stem cell-like features. Clinically, the majority of CL tumors are poor prognosis triple negative (ER-, PR-, and HER2-) invasive carcinomas with high frequencies of metaplastic and medullary differentiation(23-25). To determine whether our mouse genetic analysis models human breast cancers, we queried the expression of *BRCA1* and EMT-TFs in the UNC337 breast cancer patient sample sets(23). We found that expression of *BRCA1* and EMT-TFs were highly correlated with breast tumor intrinsic subtypes (Figure 6A). Specifically, the mRNA level of *BRCA1* was low while that of EMT-TFs – *TWIST*, *SNAIL*, and *SLUG* in particular – was high in the CL subtype. Pearson correlation analysis revealed an inverse correlation between *BRCA1* with *TWIST* mRNA levels, but not with *SNAIL*, *SLUG*, and *FOXC1* (Fig. 6B). We performed similar analyses on the MetaBric dataset with 2,000 breast tumors(26) and detected similar results – *BRCA1* and *TWIST* mRNA levels were inversely correlated in all breast cancers and in the CL subtype in particular (data not shown).

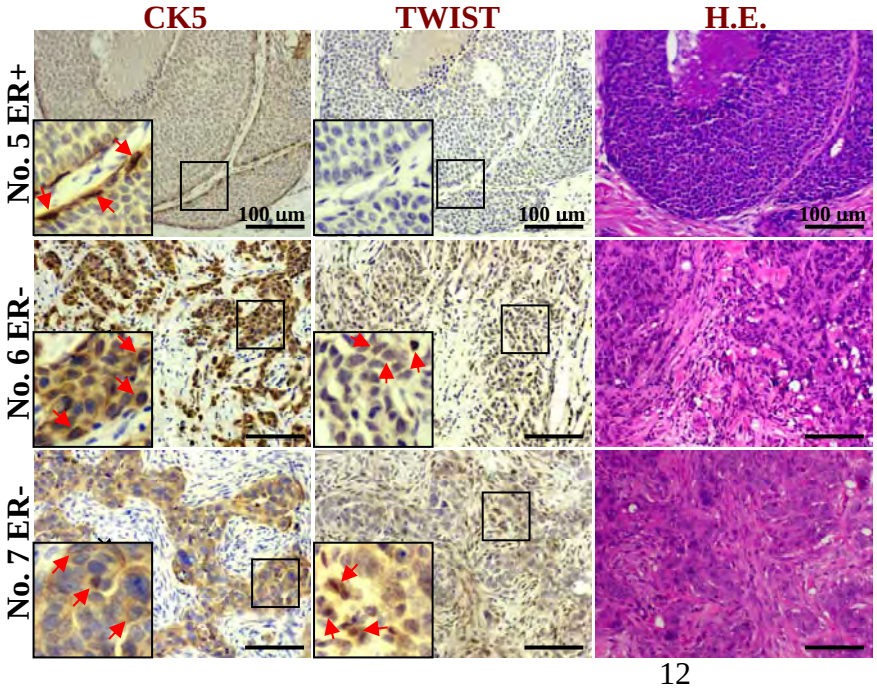
A



B

	Correlation with BRCA1 Coefficient	Correlation with BRCA1 p value
TWIST	-0.114	0.037
SNAIL	0.003	0.95
SLUG	-0.072	0.19
FOXC1	-0.052	0.35

C



D

Case no.	ER	CK5[a]	TWIST[a]	BRCA1[b]
1	+	+	-	1.3
2	+	-	-	1.7
3	+	-	-	2.1
4	+	-	-	2.4
5	+	-	-	2.7
6	-	++++	+++	0.7
7	-	+++	+++	0.8
8	-	+	+	1.0
9	-	++	+	1.4
10	-	+	-	1.9

[a] -; <5%
+; 5-25%
++: 25-50%
+++: 50-75%
++++; >75%

[b]Expression level relative to that of T47D cells

Figure 6. BRCA1 and TWIST levels are inversely related to the CL subtype of human breast cancer. (A) Analysis of gene expression in UNC337 breast cancer patients according to tumor subtype. (B) Correlation analysis of the expression of *BRCA1* and EMT-TFs for UNC337 breast cancer patients. (C) Serial sections from ER positive and negative invasive human breast cancers were stained with H.E., CK5, and TWIST. The boxed areas were enlarged in the insets. Representative cytoplasmic staining of CK5+ cells and nuclear staining of TWIST+ cells are indicated by arrows. (D) Summary of expression of CK5 and TWIST by IHC and *BRCA1* by Q-RT-PCR. The expression levels of CK5 and TWIST were scored by the percentage of positive tumor cells in total tumor cells. The expression of *BRCA1* was determined by Q-RT-PCR. The levels of *BRCA1* mRNA were expressed as the mean values from triplicates of the two sets of the primers.

We screened 43 invasive breast cancers then selected 5 ER positive and 5 ER negative samples. RNA was prepared from microdissected FFPE sections of tumors. The expression levels of *BRCA1* in ER negative tumors were significantly lower than in ER positive tumors (1.16 ± 0.49 versus 2.04 ± 0.56; $P=0.015$), reflecting the downregulation of *BRCA1* mRNA in ER negative tumors (Fig. 6D). IHC indicated that TWIST positive tumor cells were only detected in ER negative, CK5 positive tumors, not in ER positive tumors. The expression of TWIST was closely associated with that of CK5 and was inversely correlated with the mRNA level of *BRCA1* (Fig. 6C, D). Furthermore, all TWIST positive tumors were highly heterogeneous, poorly differentiated, and showed typical morphological EMT features, evidenced by the various degrees of whorls and clusters of spindle-shaped cells within these tumors. Together, these clinical findings, consistent with our results in mice, further confirm that BRCA1 suppresses TWIST and EMT in breast basal-like cancer development and progression.

4. Key Research Accomplishments

(1) Discovery that disrupting *Brca1* by either germline or epithelium-specific mutation in p18-deficient mice activates EMT and induces dedifferentiation of luminal stem cells, which associate closely with expansion of basal and cancer stem cells and formation of basal-like tumors [published in ref. (27)].

(2) Discovery that BRCA1 binds to the *TWIST* promoter, suppressing its activity and inhibiting EMT in mammary tumor cells [published in ref. (27)].

(3) Discovery that, in human luminal cancer cells, BRCA1 silencing is sufficient to activate TWIST and EMT and increase tumor formation. In parallel, *TWIST* expression and EMT features correlate inversely with *BRCA1* expression in human breast cancers [published in ref. (27)].

5. Conclusion

Our findings showed that BRCA1 suppressed TWIST and EMT, inhibited LSC dedifferentiation and repressed expansion of basal stem cells and basal-like tumors. Thus, our work offers the first genetic evidence that Brca1 directly suppresses EMT and LSC de-differentiation during breast tumorigenesis

6. Publications, Abstracts, and Presentations

(1) Publication:

Bai F, Chan HL, Scott A, Smith MD, Fan C, Herschkowitz JI, Perou CM, Livingstone AS, Robbins DJ, Capobianco AJ, **Pei XH***. BRCA1 suppresses epithelial-to-mesenchymal transition and stem cell dedifferentiation during mammary and tumor development. *Cancer Research.* 2014 Sep 19. pii: canres.1119.2014. [Epub ahead of print]. PMID: 25239453. ***Corresponding Author**

(2) Meeting Presentation:

A. Pei XH. Genetic analysis of the role of Brca1 in suppression of basal-like breast cancer. 2014 San Antonio Breast Cancer Symposium. December 9-13, 2014. San Antonio, TX.

B. Bai F, Chan HL, Scott A, Perou CM, and Pei XH. BRCA1 suppresses epithelial-to-mesenchymal transition in basal-like tumorigenesis. An AACR special conference-Advances in breast cancer research. October 3-6, 2013. San Diego, CA.

7. Inventions, Patents, and Licenses
Nothing to report

8. Reportable Outcomes
We found that disrupting *Brca1* in p18-deficient mice activates EMT and induces dedifferentiation of LSCs, which associate closely with expansion of basal and cancer stem cells and formation of basal-like tumors. Mechanistically, BRCA1 bound to the *TWIST* promoter, suppressing its activity and inhibiting EMT in mammary tumor cells. In human luminal cancer cells, BRCA1 silencing was sufficient to activate TWIST and EMT and increase tumor formation. In parallel, *TWIST* expression and EMT features correlated inversely with *BRCA1* expression in human breast cancers. Together, our findings showed that BRCA1 suppressed TWIST and EMT, inhibited LSC dedifferentiation and repressed expansion of basal stem cells and basal-like tumors. Thus, our work offers the first genetic evidence that Brca1 directly suppresses EMT and LSC de-differentiation during breast tumorigenesis.

9. Other Achievements
(1) Generation of p18;Brca1 double mutant mouse strains that develop basal-like breast cancers with high penetrance.

(2) Development of human breast cancer cell lines, of T47D-sh-Ctrol. and T47D-sh-BRCA1, which develop luminal and basal-like tumors, respectively, in mice.

10. References

1. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, et al. Distinct stem cells contribute to mammary gland development and maintenance. Nature. 2011;479(7372):189-93.
2. van Amerongen R, Bowman AN, Nusse R. Developmental Stage and Time Dictate the Fate of Wnt/beta-Catenin-Responsive Stem Cells in the Mammary Gland. Cell Stem Cell. 2012;11(3):387-400.
3. Althuis MD, Fergenbaum JH, Garcia-Closas M, Brinton LA, Madigan MP, Sherman ME. Etiology of hormone receptor-defined breast cancer: a systematic review of the literature. Cancer Epidemiol Biomarkers Prev. 2004;13(10):1558-68.
4. Arnes JB, Brunet JS, Stefansson I, Begin LR, Wong N, Chappuis PO, et al. Placental cadherin and the basal epithelial phenotype of BRCA1-related breast cancer. Clin Cancer Res. 2005;11(11):4003-11.
5. Foulkes WD, Brunet JS, Stefansson IM, Straume O, Chappuis PO, Begin LR, et al. The prognostic implication of the basal-like (cyclin E high/p27 low/p53+/glomeruloid-microvascular-proliferation+) phenotype of BRCA1-related breast cancer. Cancer Res. 2004;64(3):830-5.
6. Lakhani SR, Reis-Filho JS, Fulford L, Penault-Llorca F, van der Vijver M, Parry S, et al. Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. Clin Cancer Res. 2005;11(14):5175-80.
7. Ribeiro-Silva A, Ramalho LN, Garcia SB, Brandao DF, Chahud F, Zucoloto S. p63 correlates with both BRCA1 and cytokeratin 5 in invasive breast carcinomas: further evidence for the pathogenesis of the basal phenotype of breast cancer. Histopathology. 2005;47(5):458-66.
8. Pei XH, Bai F, Smith MD, Usary J, Fan C, Pai SY, et al. CDK inhibitor p18(INK4c) is a downstream target of GATA3 and restrains mammary luminal progenitor cell proliferation and tumorigenesis. Cancer Cell. 2009;15(5):389-401. PMCID: 2699569.
9. Bai F, Smith MD, Chan HL, Pei XH. Germline mutation of Brca1 alters the fate of mammary luminal cells and causes luminal-to-basal mammary tumor transformation. Oncogene. 2013;32(22):2715-25.
10. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420-8. PMCID: 2689101.
11. Shafee N, Smith CR, Wei S, Kim Y, Mills GB, Hortobagyi GN, et al. Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. Cancer Res. 2008;68(9):3243-50. PMCID: 2929908.

12. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, et al. Generation of a functional mammary gland from a single stem cell. Nature. 2006;439(7072):84-8.
13. Wright MH, Robles AI, Herschkowitz JI, Hollingshead MG, Anver MR, Perou CM, et al. Molecular analysis reveals heterogeneity of mouse mammary tumors conditionally mutant for Brca1. Mol Cancer. 2008;7:29. PMCID: 2329667.
14. Buono KD, Robinson GW, Martin C, Shi S, Stanley P, Tanigaki K, et al. The canonical Notch/RBP-J signaling pathway controls the balance of cell lineages in mammary epithelium during pregnancy. Dev Biol. 2006;293(2):565-80.
15. Wagner KU, Wall RJ, St-Onge L, Gruss P, Wynshaw-Boris A, Garrett L, et al. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res. 1997;25(21):4323-30. PMCID: 147032.
16. Lim E, Wu D, Pal B, Bouras T, Asselin-Labat ML, Vaillant F, et al. Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. Breast Cancer Res. 2010;12(2):R21. PMCID: 2879567.
17. Wagner KU, McAllister K, Ward T, Davis B, Wiseman R, Hennighausen L. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res. 2001;10(6):545-53.
18. Weigelt B, Kreike B, Reis-Filho JS. Metaplastic breast carcinomas are basal-like breast cancers: a genomic profiling analysis. Breast Cancer Res Treat. 2009;117(2):273-80.
19. Keller PJ, Lin AF, Arendt LM, Klebba I, Jones AD, Rudnick JA, et al. Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines. Breast Cancer Res. 2010;12(5):R87. PMCID: 3096980.
20. Shakya R, Reid LJ, Reczek CR, Cole F, Egli D, Lin CS, et al. BRCA1 tumor suppression depends on BRCT phosphoprotein binding, but not its E3 ligase activity. Science. 2011;334(6055):525-8.
21. Buckley NE, Mullan PB. BRCA1 - Conductor of the Breast Stem Cell Orchestra: The Role of BRCA1 in Mammary Gland Development and Identification of Cell of Origin of BRCA1 Mutant Breast Cancer. Stem Cell Rev. 2012.
22. Tkocz D, Crawford NT, Buckley NE, Berry FB, Kennedy RD, Gorski JJ, et al. BRCA1 and GATA3 corepress FOXC1 to inhibit the pathogenesis of basal-like breast cancers. Oncogene. 2011.
23. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res. 2010;12(5):R68. PMCID: 3096954.
24. Herschkowitz JI, Zhao W, Zhang M, Usary J, Murrow G, Edwards D, et al. Comparative oncogenomics identifies breast tumors enriched in functional tumor-initiating cells. Proc Natl Acad Sci U S A. 2012;109(8):2778-83. PMCID: 3286979.
25. Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci U S A. 2010;107(35):15449-54. PMCID: 2932589.
26. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature. 2012;486(7403):346-52. PMCID: 3440846.
27. Bai F, Chan HL, Scott A, Smith MD, Fan C, Herschkowitz JI, et al. BRCA1 suppresses epithelial-to-mesenchymal transition and stem cell dedifferentiation during mammary and tumor development. Cancer Res. 2014.

11. Appendices

Reference 27 was a direct result of this award and is attached.

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Molecular and Cellular Pathobiology

Cancer Research

BRCA1 Suppresses Epithelial-to-Mesenchymal Transition and Stem Cell Dedifferentiation during Mammary and Tumor Development

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Abstract

BRCA1 mutation carriers are predisposed to developing basal-like breast cancers with high metastasis and poor prognosis. Yet, how BRCA1 suppresses formation of basal-like breast cancers is still obscure. Deletion of $p18^{Ink4c}$ (*p18*), an inhibitor of CDK4 and CDK6, functionally inactivates the RB pathway, stimulates mammary luminal stem cell (LSC) proliferation, and leads to spontaneous luminal tumor development. Alternately, germline mutation of *Brca1* shifts the fate of luminal cells to cause luminal-to-basal mammary tumor transformation. Here, we report that disrupting *Brca1* by either germline or epithelium-specific mutation in p18-deficient mice activates epithelial-to-mesenchymal transition (EMT) and induces dedifferentiation of LSCs, which associate closely with expansion of basal and cancer stem cells and formation of basal-like tumors. Mechanistically, BRCA1 bound to the *TWIST* promoter, suppressing its activity and inhibiting EMT in mammary tumor cells. In human luminal cancer cells, BRCA1 silencing was sufficient to activate TWIST and EMT and increase tumor formation. In parallel, *TWIST* expression and EMT features correlated inversely with *BRCA1* expression in human breast cancers. Together, our findings showed that BRCA1 suppressed TWIST and EMT, inhibited LSC dedifferentiation, and repressed expansion of basal stem cells and basal-like tumors. Thus, our work offers the first genetic evidence that Brca1 directly suppresses EMT and LSC dedifferentiation during breast tumorigenesis. *Cancer Res; 74(21); 6161–72. ©2014 AACR.*

Introduction

Mammary luminal and basal epithelial cells originate from multipotent progenitors in the embryo (1–2), and expansion and maintenance of these cells in adults are ensured by unipotent luminal stem cells (LSC) and basal stem cells (BSC), respectively (1, 3). Cancer stem cells (CSC) are a subpopulation of cancer cells that shares characteristics with stem cells such as self-renewal ability and mutipotency. CSCs could generate daughter cells, thus contributing to tumor growth, and are associated with radioresistance and chemoresistance, metastasis, and poor prognosis (4). Germline mutations in the tumor suppressor *BRCA1* contribute to about half of familial breast cancer cases and increase the risk of developing basal-like breast tumors with high metastasis and poor prognosis. Basal-like tumors developed in *BRCA1* mutation carriers were thought to originate from either mammary stem cells or basal progenitors (5, 6). Recently, we and others discovered that aberrant LSCs, not BSCs/progenitors, are likely the origin of basal-like tumors developed in patients harboring *BRCA1* mutations as well as in germline $Brca1^{+/-}$-mutant mice (7–10). Furthermore, breast cancer stem cells are enriched in human *BRCA1*-mutant breast cancers (11, 12). However, whether BRCA1 functions in LSCs to maintain their unipotency, and whether and how BRCA1 controls breast cancer stem cells and basal-like tumors *in vivo*, remain elusive.

Epithelial-to-mesenchymal transition (EMT) plays an important role in intratumoral heterogeneity, breast basal-like tumor development, and generation of breast epithelial and cancer cells with stem cell–like characteristics, directly linking EMT with the gain of stem cell properties (13, 14). A set of transcription factors, including TWIST1/2, SNAIL, SLUG, ZEB1/2, and FOXC1/2, was identified as EMT-inducing transcription factors (EMT-TF). Among these, TWIST1 (TWIST) is a master regulator of EMT and plays an essential role in tumor metastasis (15).

p18 is a member of the INK4 family of cell-cycle inhibitors that inhibits CDK4/6 and, when activated by D-type cyclins,

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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phosphorylates and functionally inactivates RB, p107, and p130. *p18* expression is significantly lower in human breast cancers than normal breast (refs. 16, 17; F. Bai, unpublished data). *RB* has been identified as a major target for genomic disruption in basal-like breast cancers of *BRCA1* mutation carriers (18), and loss of both RB and BRCA1 is, indeed, a feature of basal-like human breast cancers (17, 19). Deletion of Rb alone in mouse mammary epithelia does not induce tumors (20), and deletion of both Rb and p107 results in luminal type tumors (19), suggesting a role of the Rb pathway in controlling luminal tumorigenesis. Interestingly, deletion of *p18*, which functionally inactivates the Rb pathway, stimulates mammary LSC proliferation and results in luminal type tumors (16) as well as rescues the premature senescence caused by Brca1 deficiency. Thus, *p18*;*Brca1* double-mutant mice provide a unique mouse model with a genetically intact p53 pathway and functionally inactivated Rb pathway (10) to study the role of Brca1 in breast tumor suppression.

In this report, we used *Brca1* germline and conditional mutant mice as well as human breast cancer cells and samples to determine the function and mechanism of Brca1 in suppressing EMT and basal-like tumors.

Materials and Methods

Mice, histopathology, and IHC

The generation of *p18* and *Brca1* germline mutant mice has been described previously (10, 16). *Brca1*$^{f/f}$ and Tg(MMTV-Cre) 4Mam mice were obtained from the NCI Mouse Repository and JAX lab, respectively (21, 22). All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina and University of Miami. Histopathology and IHC were performed as described previously (10, 16). Primary antibodies used are as follows: Ck5 (Covance), Ck8 (American Research Products), Ck14, SMA (Thermo Scientific), ERα, CD29, Brca1, Gata3, Foxc1, Foxc2 (Santa Cruz), E-cadherin (BD Biosciences), fibronectin, vimentin, Twist, Snail (Abcam), and Slug (Novus Biologicals).

Mammary cell preparation, FACS analysis, cell sorting, mammosphere assay, colony-formation assay, cell lines, transfection, and lentiviral infection

Mammary glands were dissected from female mice at the indicated ages, and the tissue was processed as previously described (10, 16, 23, 24). MCF-7, T47D (ATCC), SUM149 (Dr. Sendurai Mani, University of Texas, Houston, TX), and HCC1937 (Dr. Jennifer Hu, University of Miami, Miami, FL) cells were tested and authenticated (10, 25, 26). For ectopic expression of BRCA1, HCC1937 cells were transfected with pcDNA3-empty or pcDNA3-BRCA1 with FuGene. For BRCA1 knockdown (KD), pGIPZ-empty (Sh-Ctrl) and pGIPZ-shBRCA1 (Sh-BRCA1) lentiviral vectors were purchased from Open Biosystems.

Xenograft models of breast cancer

T47D and MCF-7 Sh-Ctrl and Sh-BRCA1 cells were suspended in a 50% solution of Matrigel (BD), and then inoculated into the left and right inguinal mammary fat pads of 6-week-old female NSG mice (Jackson Laboratory), respectively. Eighteen weeks after transplantation, animals were euthanized and mammary tumors were dissected for analyses. No estrogen was administered to animals during the course of the study.

Western blot, qRT-PCR, and chromatin immunoprecipitation assay

Western blot, QRT-PCR, and chromatin immunoprecipitation (ChIP) assay were carried out as previously described (10, 16, 27). Primary antibodies used for Western blot are as follows: Brca1, Gata3 (Santa Cruz), E-cadherin, fibronectin, tubulin-α (DM1A; NeoMarkers), and actin (ACTN05; NeoMarkers). Anti-BRCA1 antibody (D-9; Santa Cruz) or control mouse IgG was used to precipitate chromatin associated with BRCA1. qPCR was performed to determine the relative abundance of target DNA. Specific primers for the analysis of BRCA1 binding to *TWIST* are available upon request.

Human tumor samples

Formalin-fixed paraffin-embedded (FFPE) human breast cancer samples lacking patient-identifying information were obtained from the Tissue Bank Core Facility at the University of Miami. All samples obtained were nontreated invasive breast cancers with known estrogen receptor (ER) status. Regions from tumor samples were microdissected, and only samples with a consistent tumor cell content >75% of tissues were used for RNA extraction. The expression of *BRCA1* was determined by qRT-PCR.

Patients and gene-expression datasets

The UNC337 human breast cancer dataset (28) with 337 breast cancer samples and the MetaBric dataset (29) with 2,000 samples were analyzed. We compared gene expression versus six breast cancer subtypes using two-way ANOVA.

Results

Germline mutation of *Brca1* transforms *p18*$^{-/-}$ luminal tumors into basal-like tumors with induction of EMT

In our previous studies, we reported that deletion of *p18* in mice stimulates mammary LSC proliferation and leads to spontaneous luminal tumor development (16), and that germline mutation of *Brca1* in p18-deficient mice blocks the expansion of LSCs and transforms luminal tumors into basal-like tumors (10). Prompted by the highly invasive heterogeneous mammary tumors developed in *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice with various degrees of whorls and clusters of spindle-shaped cells within these tumors—typical morphologic characteristics of mesenchymal cells (10)—we looked at molecular markers associated with EMT. We found that the majority of the luminal tumors from *p18*$^{-/-}$ mice highly expressed E-cadherin (Cdh1), an epithelial marker, whereas basal-like tumors from *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice expressed very weak and heterogeneous Cdh1. In contrast, most (77%, $n = 13$) of *p18*$^{-/-}$;*Brca1*$^{+/-}$ tumors that developed after one year of age were stained positive for mesenchymal markers, including fibronectin (Fn), vimentin (Vim), and CD29, whereas only 11% ($n = 19$) of *p18*$^{-/-}$

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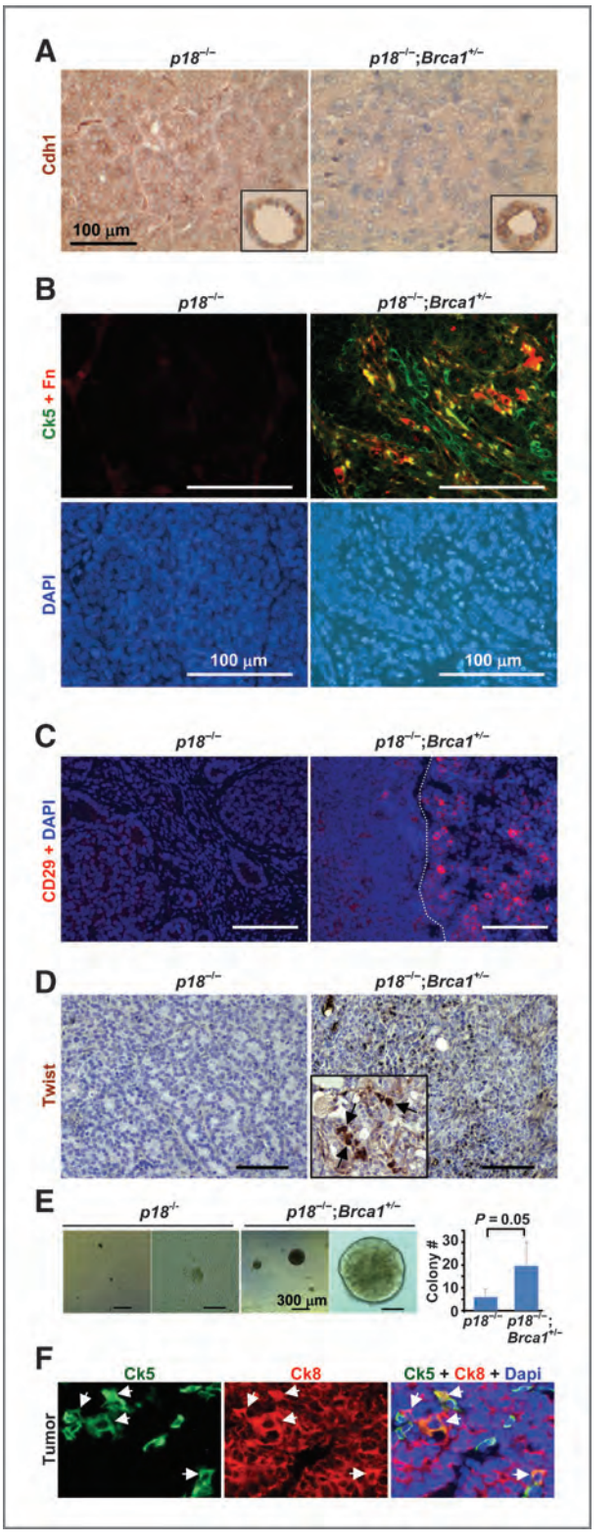

Figure 1. *Brca1* heterozygosity transforms p18-deficient luminal tumors into basal-like tumors with EMT features. A–D, representative immunostaining of tumors with Cdh1 (A), Ck5 and Fn1 (B), CD29 (C), and Twist (D). The inset in A shows staining of normal glands in the same mouse and in D shows staining of lung metastasis. E, tumor cells were cultured for 2 weeks, and colonies larger than 30 μm were counted. Bar graph, mean ± SD of two tumors per genotype. F, representative immunostaining of tumors from $p18^{-/-}$;$Brca1^{+/-}$ mice with Ck5 and Ck8. $Ck5^{+}$ $Ck8^{+}$ cells are indicated.

tumors that developed at a similar age were positive for these markers (Fig. 1A–C; Supplementary Fig. S1A–S1D; Table 1). This observation suggests that heterozygous germline mutation of *Brca1* activates EMT in mammary tumor progression.

Consistently, $p18^{-/-}$;$Brca1^{+/-}$ tumor cells that were positive for Ck5 expressed very low levels of Cdh1 (Supplementary Fig. S1A) and the majority of Fn-positive cells coexpressed Ck5 (Fig. 1B; Supplementary Fig. S1C). These data suggest, at the least, that some $Ck5^{+}$ basal-like tumor cells lost their epithelial characteristics and gained mesenchymal features. In further analysis of these tumors for the expression of CD29, a basal and mesenchymal marker (14) demonstrated to be enriched in breast CSCs (23, 30), we found that 69% ($n = 13$) of $p18^{-/-}$;$Brca1^{+/-}$ tumors expressed various degrees of CD29-positive tumor cells from 2% to 60%, whereas only 11% ($n = 19$) of $p18^{-/-}$ tumors were positive for CD29 in 2% to 3% of tumor cells (Fig. 1C and Table 1). These observations support the notion that EMT activation, as previously demonstrated (13, 14), results in cancer cells gaining stem cell properties. Primary $p18^{-/-}$;$Brca1^{+/-}$ tumor cells formed more and larger colonies in Matrigel than $p18^{-/-}$ tumor cells (Fig. 1E), and Ck5/Ck8 double-positive tumor cells were frequently detected in $p18^{-/-}$;$Brca1^{+/-}$ tumors, but rarely in $p18^{-/-}$ tumors, 1.1% (67/6,100) versus 0.04% (2/5,120; Fig. 1F; Supplementary Fig. S1E and refs. 10, 16), which further suggests increased CSCs in $p18^{-/-}$;$Brca1^{+/-}$ tumors. Together, these results indicate that heterozygous germline mutation of *Brca1* induces EMT, increases CSCs, and transforms p18 null luminal tumors into basal-like tumors.

Germline mutation of *Brca1* activates EMT-TFs in mammary and tumor development

We then determined the expression of EMT-TFs and observed that 77% ($n = 13$, >1 year of age) of $p18^{-/-}$;$Brca1^{+/-}$ tumors were stained positive for Twist, Foxc1, Foxc2, Slug, and Snail in greater than 2% of cells per tumor, whereas 16% ($n = 19$, >1 year of age) of $p18^{-/-}$ tumors were positive at similar ages (Fig. 1D; Supplementary Fig. S2A–S2C; and Table 1). Tumors with high expression of EMT-TFs showed high histologic grade and strong invasive and metastatic potential as evidenced by EMT-TF–positive staining in the invasive front of tumors and metastasized cancers (Fig. 1D). The expression pattern and percentage of positive cells in tumors stained for EMT-TFs and EMT markers were highly correlated with its genotype—$p18^{-/-}$ or $p18^{-/-}$;$Brca1^{+/-}$—which not only confirms that germline mutation of *Brca1* promotes EMT in mammary tumors but that this induction of EMT is very likely a result of the aberrant activation of EMT-TFs in *Brca1*-mutant tumors. We next isolated mammary epithelial cells (MEC) from tumor-free virgin mice and found that $Brca1^{+/-}$ and $p18^{-/-}$;$Brca1^{+/-}$ cells expressed significantly less *Cdh1* and more EMT-TFs than wild-type (WT) or $p18^{-/-}$ cells (Supplementary

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Table 1. Characterization of spontaneous mammary tumors derived from mutant mice

	WT		$p18^{-/-}$		$Brca1^{+/-}$		$p18^{-/-}$;$Brca1^{+/-}$	
Tumor	**<12 mo**	**12–27 mo**	**<12 mo**	**12–22 mo**	**<12 mo**	**12–27 mo**	**<12 mo**	**12–22 mo**
Mammary tumor	0/5	1/10[a] (10%)	4/16 (25%)	19/23[b] (83%)	0/3	1/11[c] (9%)	6/16 (38%)	13/15[d] (87%)
Metastasis[e]		0/1	0	1/19		0/1	0	4/13
ERα$^+$ tumor		1/1	3/4	15/19		0/1	1/6	2/13
% ERα$^+$ cells/tumor		5%	2%–40%	2%–40%			<2%	<2%
Ck5$^+$ tumor		0/1	0/4	3/19[f]		1/1	4/6	11/13[g]
%Ck5$^+$ cells/tumor				1%–5%		∼2%	2%–20%	2%–95%
EMT marker$^+$ tumor[h]		0/1	0/4	2/19 (11%)		0/1	2/6 (33%)	10/13 (77%)
EMT-TF$^+$ tumor[i]		0/1	0/4	3/19 (16%)		1/1 (100%)	3/6 (50%)	10/13 (77%)

[a]24-month-old tumor-bearing mouse.
[b]Most tumor-bearing mice were 12 to 16 months old, and the oldest was 22 months old. One male developed mammary tumor.
[c]25.5-month-old tumor-bearing mouse.
[d]Most tumor-bearing mice were 12 to 16 months old, and the oldest was 20 months old. One male developed mammary tumor.
[e]Mammary tumors metastasized mostly to the lung except one to a blood vessel in a $p18^{-/-}$;$Brca1^{+/-}$ mouse.
[f]One tumor stained positive for Ck5 in approximately 5% tumor cells and the other two were positive in approximately 1% tumor cells.
[g]Two tumors stained positive for Ck5 in approximately 95% tumor cells.
[h]At least two EMT markers (decreased Cdh1, increased Vim, Fn1, Sma, or Cd29) were detected in >2% tumor cells.
[i]At least two EMT-TFs, which include Twist, Slug, Snail, Foxc1, and Foxc2, stained positive in >2% tumor cells.

Fig. S2D). These results indicate that EMT-TF activation in *Brca1*-mutant MECs occurs before tumor initiation.

Specific deletion of *Brca1* in mammary epithelia activates EMT and induces aberrant differentiation of LSCs

To directly test the function of Brca1 in controlling and transforming MECs as well as to determine the implications of loss of Brca1 on mammary tumorigenesis, we generated $Brca1^{f/f}$;MMTV-cre$^+$ and $Brca1^{f/-}$;MMTV-cre$^+$ mice with and without *p18* mutation, in which MMTV-cre (MC) is active in virgin epithelia but not in stroma (22, 31). Using these mice also enabled us to rule out the impact of *Brca1*-mutant stroma on mammary stem cell self-renewal and tumorigenesis.

$Brca1^{f/-}$;MC and $p18^{-/-}$;$Brca1^{f/-}$;MC breasts expressed <5% of Brca1 protein and mRNA relative to the levels in $Brca1^{f/+}$;MC and $p18^{-/-}$;$Brca1^{f/+}$;MC, indicating an efficient and near complete depletion of Brca1 in the mammary epithelia (Fig. 2A and B; Supplementary Fig. S3). Similarly, $Brca1^{f/f}$;MC and $p18^{-/-}$;$Brca1^{f/f}$;MC breasts expressed <20% of Brca1 protein and mRNA relative to the levels in MC and $p18^{-/-}$;MC (data not shown). Consistent with the data from $Brca^{+/-}$ mice (10), the expression of *Gata3*, *Cdh1*, and *Epcam*—genes associated with luminal differentiation—in $Brca1^{f/-}$;MC and $p18^{-/-}$;$Brca1^{f/-}$;MC breasts was significantly reduced relative to $Brca1^{f/+}$;MC and $p18^{-/-}$;$Brca1^{f/+}$;MC breasts (Fig. 2A and B; Supplementary Fig. S3), suggesting that loss of Brca1 impairs luminal differentiation. MECs from $p18^{-/-}$;$Brca1^{f/-}$;MC mice showed increased mammosphere-forming ability than those from $p18^{-/-}$;$Brca1^{f/+}$;MC mice. Most $p18^{-/-}$;$Brca1^{f/+}$;MC mammospheres were 35 to 45 μm and none larger than 100 μm, whereas 10% to 15% of $p18^{-/-}$;$Brca1^{f/-}$;MC mammospheres were larger than 100 μm. The average $p18^{-/-}$;$Brca1^{f/-}$;MC mammosphere was significantly larger than that of $p18^{-/-}$;$Br^{f/+}$;MC mammospheres (Fig. 2C). These results suggest that Brca1 deficiency increased the self-renewal capacity of $p18^{-/-}$ mammary stem cells. Accordingly, MECs from $p18^{-/-}$;$Brca1^{f/-}$;*MC* mice formed more colonies than those from $p18^{-/-}$;$Brca1^{f/+}$;MC mice and $p18^{-/-}$;$Brca1^{f/-}$;MC mammospheres expressed significantly higher levels of EMT-TFs than those of $p18^{-/-}$;$Brca1^{f/+}$;MC (Fig. 2D and E). These results confirm that loss of Brca1 activates EMT-TFs, which is likely responsible for the induction of EMT and increased mammosphere- and colony-forming potential in $p18^{-/-}$;$Brca1^{f/-}$;MC MECs.

We then performed FACS and found that $p18^{-/-}$;$Brca1^{f/-}$;MC MECs had a reduced CD24$^+$CD29$^-$ LSC-enriched population and increased CD24$^+$CD29$^+$ BSC-enriched population compared with $p18^{-/-}$;$Brca1^{f/+}$;MC MECs at 22 weeks of age (Fig. 2F). Similar, but less significant, trends were also observed in $p18^{-/-}$;$Brca1^{f/+}$;MC mice relative to $p18^{-/-}$;MC mice at 16 weeks of age (Supplementary Fig. S4A and S4B). These results suggest that Brca1 deficiency results in the expansion of BSCs and blockage of LSCs, the latter of which is consistent with our findings derived from heterozygous germline *Brca1*-mutant mice (10).

FACS-sorted cells of the BSC-enriched population expressed higher basal genes (*Twist2*, *Id4*, and *Tbx2*) and lower luminal

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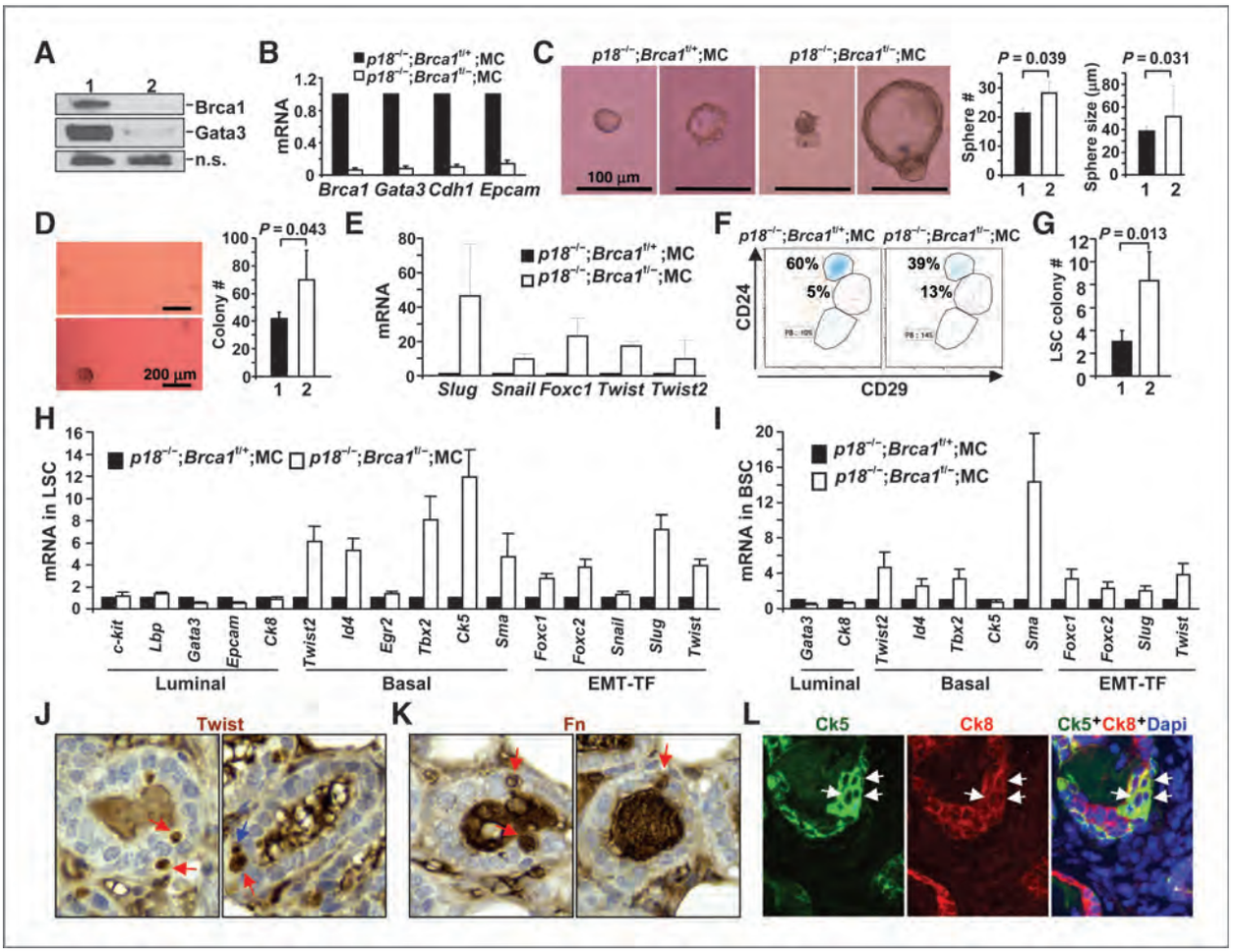

Figure 2. Deletion of Brca1 in mammary epithelia inhibits luminal differentiation and activates EMT-TFs in mammary stem cells. A and B, mammary tissues from $p18^{-/-}$;$Brca1^{f/+}$;MC (lane 1) and $p18^{-/-}$;$Brca^{f/-}$;MC (lane 2) mice were analyzed by Western blot (A) and qRT-PCR (B). n.s., non-specific band. qRT-PCR data are expressed as the mean ± SD from triplicates of each of three separate mice. C and D, mammary cells were analyzed by mammosphere (C) and colony-formation assay (D). The number of spheres larger than 30 μm, the sizes of spheres, and the number of colonies larger than 30 μm were quantified. 1, $p18^{-/-}$;$Brca1^{f/+}$;MC; 2, $p18^{-/-}$;$Brca1^{f/-}$;MC. The bar graphs represent the mean ± SD of two animals per genotype. E, RNA from mammospheres was analyzed. Data are expressed as mean ± SD from triplicates of each of two separate mice. F, mammary cells were analyzed by flow cytometry. G, FACS-sorted LSCs from F were analyzed by colony-formation assay. The bar graphs represent the mean ± SD of two animals per genotype. 1, $p18^{-/-}$;$Brca1^{f/+}$;MC; 2, $p18^{-/-}$;$Brca1^{f/-}$;MC. H and I, RNA from LSCs (H) and BSCs (I) was analyzed. Data are expressed as the mean ± SD from triplicates of each of two separate mice. J–L, tumor-free mammary glands from $p18^{-/-}$;$Brca1^{f/-}$;MC mice were stained with antibodies against Twist (J), Fn (K), Ck5 and Ck8 (L). Twist or Fn-positive ULLC (red arrows) and SLC (blue arrows), as well as $Ck5^+$ $Ck8^+$ epithelial cells (white arrows), are indicated.

genes (*c-kit*, *Epcam*, and *Gata3*) than those of the LSC-enriched population, confirming that these cell populations are, as reported (32), the basal and luminal cell–enriched populations, respectively (Supplementary Fig. S4D). LSCs derived from $p18^{-/-}$;$Brca1^{f/-}$;MC mice formed more colonies in Matrigel and expressed lower luminal and epithelial genes and significantly higher basal genes and EMT-TFs when compared with *p18*;$Brca1^{f/+}$;MC LSCs (Fig. 2G and H). Consistently, LSCs from $p18^{-/-}$;$Brca1^{f/+}$;MC mice also expressed lower luminal genes and higher basal genes and EMT-TFs than those from $p18^{-/-}$; MC mice (Supplementary Fig. S4C). These results indicate that haploid or near complete loss of *Brca1* in mammary epithelium not only inhibits the expression of luminal genes but also stimulates the expression of basal genes and EMT-TFs in $p18^{-/-}$ LSCs. Interestingly, expression of basal genes and EMT-TFs was also significantly increased in the BSCs from $p18^{-/-}$;$Brca1^{f/-}$;MC mice relative to those from *p18*;$Brca1^{f/+}$; MC mice (Fig. 2I). Together, these results suggest that Brca1 deficiency leads to the expansion of BSCs, which is likely, at least partially, a result of the dedifferentiation of LSCs.

We have previously analyzed five histologically distinct epithelial cell populations and defined the small light cell (SLC) and undifferentiated large light cell (ULLC) populations as enriched for stem and luminal stem/progenitor cells, respectively (16). To determine the impact of EMT on stem/progenitor cell populations *in situ*, we examined tumor-free mammary glands and found that Twist or Fn-positive MECs were frequently detected in $p18^{-/-}$;$Brca1^{f/-}$;MC or $p18^{-/-}$;$Brca1^{f/f}$;MC

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mice but not in $p18^{-/-}$;MC mice and that most, if not all, Twist or Fn-positive cells were either SLC or ULLC, ULLC in particular (Fig. 2J and K). Furthermore, Ck5 and Ck8 double-positive epithelial cells were also frequently detected in $p18^{-/-}$;$Brca1^{f/-}$;MC but not in $p18^{-/-}$;MC mammary (Fig. 2L; Supplementary Fig. S5; data not shown). These results further suggest that loss of Brca1 in MECs activates Twist, induces EMT, and leads to dedifferentiation of LSCs.

Specific deletion of Brca1 in mammary epithelia recapitulates basal-like tumorigenesis and EMT activation

To determine the tumorigenic impact of specific loss of Brca1 in mammary epithelia, we first examined $Brca1^{f/-}$;MC and $p18^{-/-}$;$Brca1^{f/-}$;MC mice and found that no hyperplasia nor tumors developed in 5 female $Brca1^{f/-}$;MC mice at 10 to 12 months of age. Of the 8 $p18^{-/-}$;$Brca1^{f/-}$;MC mice examined at similar ages, all developed mammary hyperplasia, though no mammary tumors, were detected. A majority (7/8) of $p18^{-/-}$;$Brca1^{f/-}$;MC mice died at early ages from carcinomas in the pancreas, skin, pituitary, or lung (data not shown), very likely due to active MMTV-Cre expression and near complete deletion of Brca1 in these tissues (33), which prevented us from observing the relatively late-onset mammary tumorigenesis in these mice. These results, however, confirm the previous findings that loss of Brca1 alone is insufficient to promote tumorigenesis and that Brca1 cooperates with p18 to control tumorigenesis.

We then examined $p18^{-/-}$;$Brca1^{f/+}$;MC and $p18^{-/-}$;$Brca1^{f/f}$;MC mice and found that 1 of 4 $p18^{-/-}$;$Brca1^{f/+}$;MC mice and 4 of 5 $p18^{-/-}$;$Brca1^{f/f}$;MC mice developed mammary tumors in 12 to 16 months (Fig. 3). In accordance with the tumors developed in $p18^{-/-}$;$Brca1^{+/-}$ mice, mammary tumors in $p18^{-/-}$;$Brca1^{f/+}$;MC and $p18^{-/-}$;$Brca1^{f/f}$;MC mice were also highly heterogeneous, poorly differentiated, and more aggressive than those developed in $p18^{-/-}$ mice (Figs. 1, 3; Supplementary Fig. S1 and S2). About 25% to 30% $p18^{-/-}$;$Brca1^{f/+}$;MC tumor cells were spindle-shaped and were positive for Twist and Fn (Fig. 3A and B), and more than 40% of the tumor cells were positive for Ck5 and negative for Cdh1 or Ck8 (Fig. 3C and D), indications of a basal-like tumor undergoing EMT. The $p18^{-/-}$;$Brca1^{f/+}$;MC mammary tumors also expressed 1/3 of *Brca1* and 1/5 of *Gata3* relative to the tumor-free mammary tissues of the same mouse (Fig. 3E), confirming deficient Brca1 and downregulation of Gata3 in the tumor.

More than 25% of tumor cells were spindle-shaped in all four $p18^{-/-}$;$Brca1^{f/f}$;MC mammary tumors, and two displayed more than 90% spindle-shaped cells (Fig. 3F). These tumors were also positive for Twist and Fn (Fig. 3G), indications of typical metaplastic breast carcinomas undergoing EMT. A $p18^{-/-}$;$Brca1^{f/f}$;MC tumor expressed less than 10% *Brca1* and *Gata3* when compared with tumor-free mammary of the same mouse (Fig. 3I). FACS showed that the LSC-enriched population in $p18^{-/-}$;$Brca1^{f/f}$;MC mammary tumors was significantly reduced in comparison with the tumor-free mammary tissues of the same mouse (6% vs. 56%) and when compared with $p18^{-/-}$ mammary tumor cells (6% vs. 57%). Contrastingly, the BSC-enriched population, also enriched with breast CSCs, was significantly expanded in $p18^{-/-}$;$Brca1^{f/f}$;MC mammary tumors relative to the tumor-free mammary tissues of the same mouse (19% vs. 11%) and when compared with $p18^{-/-}$ mammary tumor cells (19% vs. 4%; Fig. 3H). These results further support that $p18^{-/-}$;$Brca1^{f/f}$;MC mammary tumors are basal-like tumors undergoing EMT that are enriched with CSCs, which is in line with the data derived from human patients showing that metaplastic breast carcinomas are basal-like breast cancers with EMT-like molecular make-up and are closely correlated with BRCA1 dysfunction (34).

Taken together, these results suggest that insufficient Brca1 in mammary epithelial cells represses Gata3, activates Twist and EMT, and results in basal-like tumorigenesis with an increase in the CSC population. Because $p18^{-/-}$;$Brca1^{f/+}$;MC and $p18^{-/-}$;$Brca1^{f/f}$;MC mice are in B6 and Balb/c mixed backgrounds, unlike $p18^{-/-}$;$Brca1^{+/-}$ mice in pure Balb/c background, these data also suggest that the role of Brca1 controlling basal-like tumorigenesis and EMT is independent of genetic background.

BRCA1 suppresses TWIST transcription and EMT

We screened a panel of human breast cancer cell lines and found that MCF7 and T47D cells expressed higher CDH1 and GATA3 and lower VIM and EMT-TFs than SUM149 and HCC1937 cells (Supplementary Fig. S6), confirming that MCF7 and T47D cells are luminal/epithelial-like and SUM149 and HCC1937 cells are basal/mesenchymal-like cancer cells in our culture system (35). Transfection of WT *BRCA1* into HCC1937 (*BRCA1* mutant, transcriptionally null) cells resulted in increase of *CDH1* and decrease of *VIM* and *FN*, indicating that BRCA1 suppresses EMT. Importantly, ectopic expression of BRCA1 significantly repressed *TWIST* by more than 50% compared with control, moderately repressed *FOXC2*, but hardly repressed other EMT-TFs (Fig. 4A). A similar inhibitory effect on *TWIST* and *FOXC2* expression was also detected in 293T cells transfected with BRCA1 (Supplementary Fig. S7). Because the ability of BRCA1 in regulating transcription controls normal differentiation and suppresses tumor development (36, 37), we determined whether BRCA1 is recruited to the *TWIST* promoter. A previous study demonstrated that GATA3 recruits BRCA1 to its binding sites in the *FOXC1/2* promoters to repress their transcription (27). We performed bioinformatic analysis of the *TWIST* gene promoter and found that there exists, at the least, six putative GATA3 binding sites on the *TWIST* promoter (Fig. 4B), which are conserved in both human and mouse (data not shown). We then performed a ChIP assay and found that one of five amplicons that contained two GATA3 sites was specifically enriched in the immunoprecipitation of BRCA1 in HCC1937 cells transfected with WT *BRCA1* compared with control (P5 in Fig. 4C). In sum, these results suggest that BRCA1 specifically binds to the TWIST promoter and negatively regulates its transcription.

To confirm the role of Brca1 in the suppression of Twist and tumor development *in situ*, primary mammary tumors derived from $p18^{-/-}$;$Brca1^{+/-}$ mice were immunostained with antibodies against Brca1 and Twist. We found that tumor cells positive for Brca1 expressed very low or no Twist, whereas Brca1-mutant tumor cells expressed high levels of Twist, most

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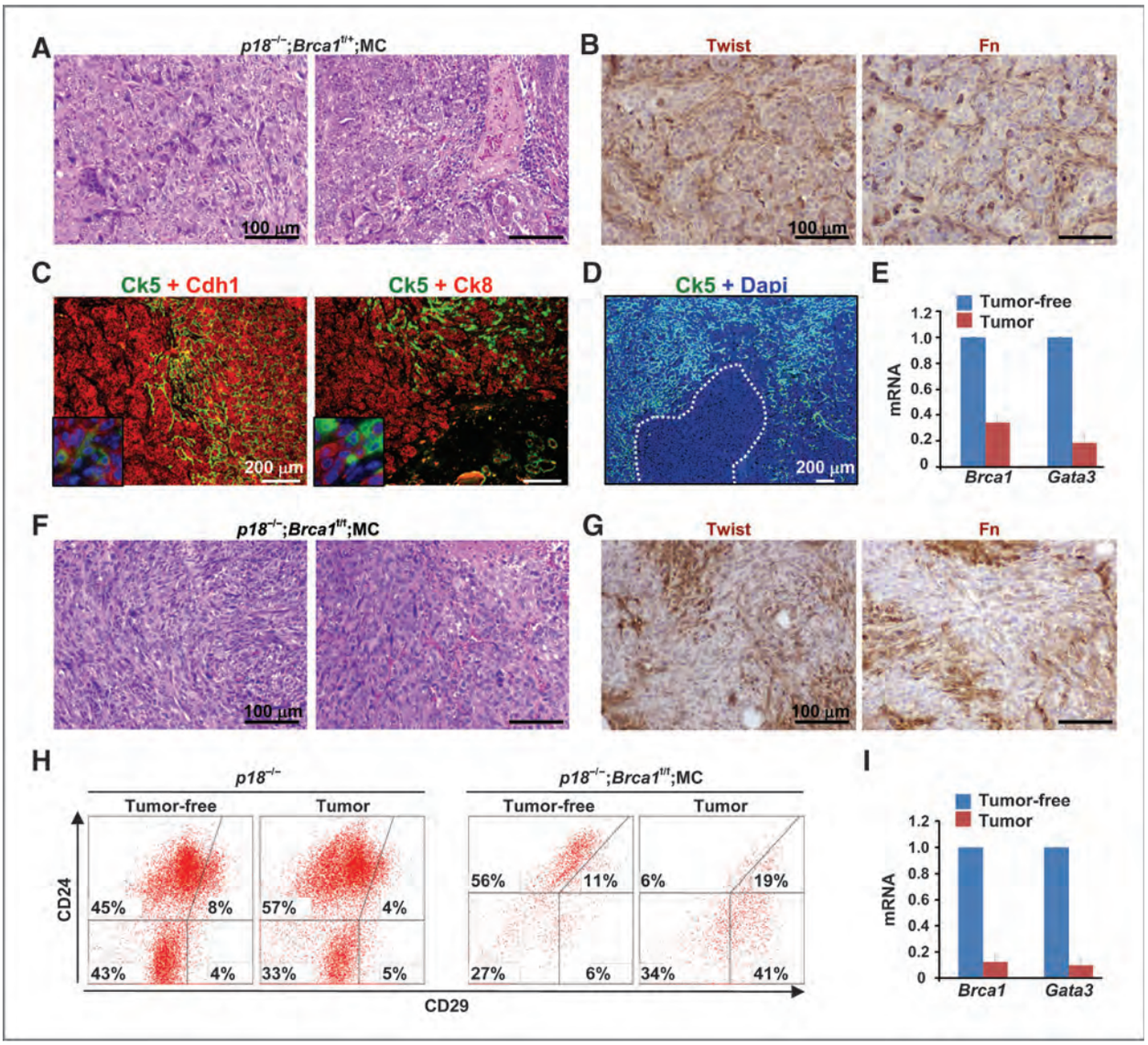

Figure 3. Deletion of Brca1 in mammary epithelia recapitulates basal-like tumor formation and EMT activation. Mammary tumors derived from $p18^{-/-}$;$Brca1^{f/+}$;MC (A–E) or $p18^{-/-}$;$Brca1^{f/f}$;MC (F–I) mice were stained with hematoxylin and eosin (A and F), Twist or Fn (B, G), Ck5 and Cdh1 or Ck8 (C), Ck5 (D), or analyzed by qRT-PCR (E, I) and FACS (H). Tumor-free mammary cells or tissues from the same mouse were used as controls. qRT-PCR data are expressed as the mean ± SD from triplicates.

of which were spindle-shaped basal-like cells (Fig. 4D), demonstrating that Brca1 inhibits Twist and EMT in mammary tumor development and progression.

Knockdown of BRCA1 inactivates TWIST and EMT with enhanced tumor formation potential

We knocked down BRCA1 in two human luminal cancer cell lines, MCF7 and T47D, using BRCA1 shRNA targeting 3 different sequences (Fig. 5A, data not shown), and transplanted these cells into the mammary fat pads of NSG mice. We found that mammary tumors from T47D-Sh-BRCA1 cells were palpable in 8 weeks, whereas tumors formed from T47D-Sh-Ctrl. cells were undetectable at this stage (data not shown). Eighteen weeks after transplantation, T47D-Sh-BRCA1 tumors were significantly bigger than T47D-Sh-Ctrl. tumors (Fig. 5B and C). Consistently, mammary tumors from MCF7-Sh-BRCA1 cells were palpable significantly sooner and were larger compared with tumors from MCF7-Sh-Ctrl. cells (data not shown). These results indicate that KD of BRCA1 in luminal cancer cells enhances their tumor formation potential. Histo- and pathologic analysis revealed that, unlike homogeneous and well-differentiated T47D-Sh-Ctrl. mammary tumors, T47D-Sh-BRCA1 tumors were highly heterogeneous with an abundance of large and poorly differentiated cells (Fig. 5D), suggesting that KD of BRCA1 induced the dedifferentiation of luminal tumor cells. IHC analysis indicated that most cells in T47D-Sh-BRCA1

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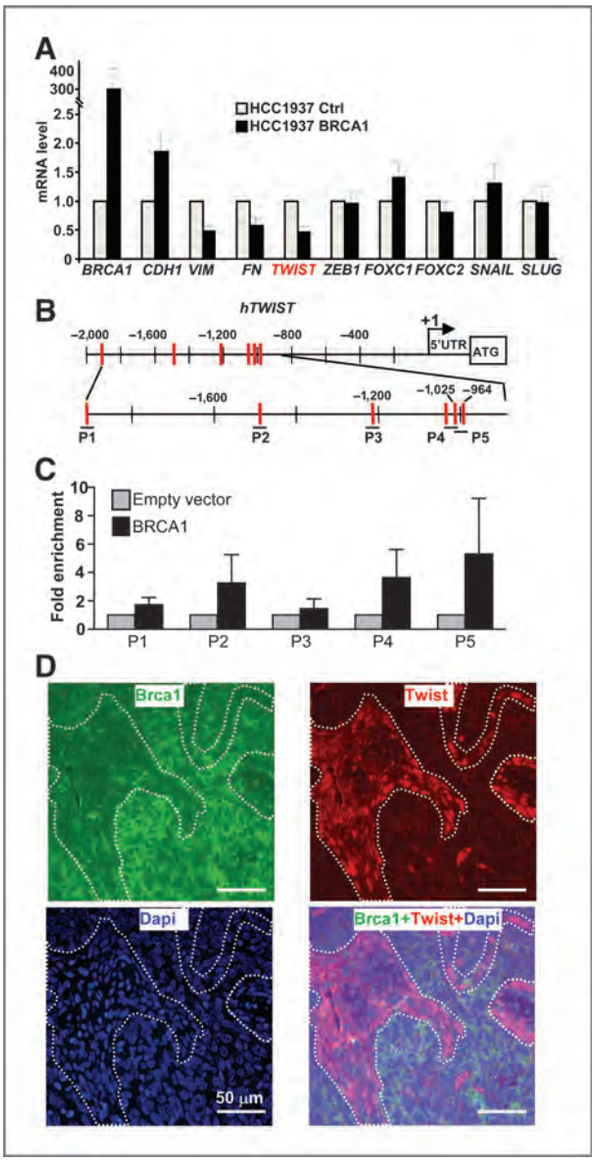

Figure 4. BRCA1 suppresses TWIST and EMT in mammary tumor cells. A, HCC1937 cells were transfected with pcDNA3-empty (Ctrl) or pcDNA3-BRCA1 (BRCA1), and RNA was analyzed. Data are expressed as the mean ± SD from triplicates of two independent experiments. B, diagram showing the locations of putative GATA3 sites in the human *TWIST* gene. C, ChIP analysis of BRCA1 binding to putative GATA3 sites on the *TWIST* promoter in HCC1937 cells transfected with *BRCA1*. Data are expressed as the mean ± SD from triplicates of two independent experiments. D, mammary tumors from $p18^{-/-}$;$Brca1^{+/-}$ mice were stained with antibodies against Brca1 (green) and Twist (red).

tumors expressed very faint or no CDH1 and ERα, but high levels of TWIST, VIM, and FN in comparison with T47D-Sh-Ctrl. tumors (Fig. 5E–G; Supplementary Fig. S8), indicating that T47D-Sh-BRCA1 tumor cells had undergone EMT. These results collectively suggest that KD of BRCA1 in breast luminal tumor cells activates TWIST and EMT, which is associated with increased tumor formation potential, further supporting the data derived from *p18*;*Brca1* double-mutant mice.

BRCA1 and *TWIST* expression levels are inversely related in human claudin-low type breast cancers

Gene-expression profiling analyses have categorized human breast tumors into six intrinsic subtypes: basal-like (BL), claudin-low (CL), $Her2^{+}$ (H2), luminal A (LA), luminal B (LB), and normal breast-like (NBL), each of which has unique biologic and prognostic features (28). Of these subtypes of breast cancer, the CL subtype is characterized by the low to absent expression of luminal differentiation markers and high enrichment for EMT markers and cancer stem cell–like features. Clinically, the majority of CL tumors are poor prognosis triple-negative (ER^{-}, PR^{-}, and $HER2^{-}$) invasive carcinomas with high frequencies of metaplastic and medullary differentiation (28, 38, 39). To determine whether our mouse genetic analysis models human breast cancers, we queried the expression of *BRCA1* and EMT-TFs in the UNC337 breast cancer patient sample sets (28). We found that expressions of *BRCA1* and EMT-TFs were highly correlated with breast tumor–intrinsic subtypes (Fig. 6A). Specifically, the mRNA level of *BRCA1* was low, whereas that of EMT-TFs—*TWIST*, *SNAIL*, and *SLUG* in particular—was high in the CL subtype. Pearson correlation analysis revealed an inverse correlation between *BRCA1* with *TWIST* mRNA levels, but not with *SNAIL*, *SLUG*, and *FOXC1* (Fig. 6B). We performed similar analyses on the MetaBric dataset with 2,000 breast tumors (29) and detected similar results—*BRCA1* and *TWIST* mRNA levels were inversely correlated in all breast cancers and in the CL subtype in particular (Supplementary Fig. S9).

We screened 43 invasive breast cancers then selected five ER-positive and five ER-negative samples. RNA was prepared from microdissected FFPE sections of tumors. The expression levels of *BRCA1* in ER-negative tumors were significantly lower than in ER-positive tumors (1.16 ± 0.49 vs. 2.04 ± 0.56; $P = 0.015$), reflecting the downregulation of *BRCA1* mRNA in ER-negative tumors (Fig. 6D). IHC indicated that TWIST-positive tumor cells were only detected in ER-negative, CK5-positive tumors, not in ER-positive tumors. The expression of TWIST was closely associated with that of CK5 and was inversely correlated with the mRNA level of *BRCA1* (Fig. 6C and D). Furthermore, all TWIST-positive tumors were highly heterogeneous, poorly differentiated, and showed typical morphologic EMT features, evidenced by the various degrees of whorls and clusters of spindle-shaped cells within these tumors. Together, these clinical findings, consistent with our results in mice, further confirm that BRCA1 suppresses TWIST and EMT in breast basal-like cancer development and progression.

Discussion

In this article, we confirm our previous finding that heterozygous mutation of *Brca1* in p18-deficient mice transforms luminal tumors into basal-like tumors (10) and report here that it activates EMT-TFs and induces EMT. Consistently, specific deletion of Brca1 in mammary epithelia led to enhanced self-renewal potential of stem cells, blockage of LSC expansion, impaired luminal gene expression, increased basal gene

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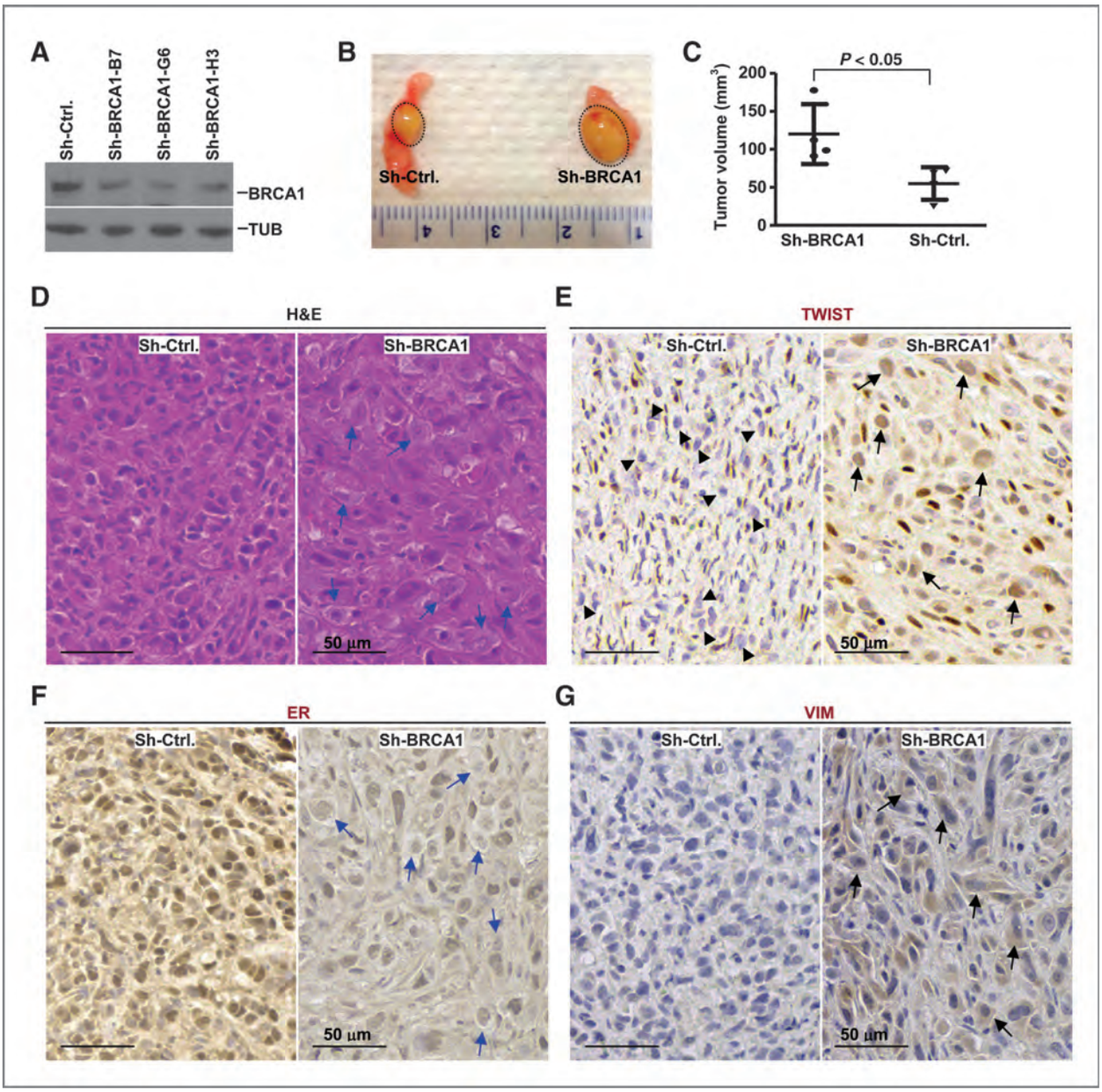

Figure 5. Knockdown of BRCA1 in luminal cancer cells increases tumor formation potential and TWIST expression. A, T47D cells were infected with either pGIPZ-empty (sh-Ctrl.) or pGIPZ-shBRCA1 targeting different sequences of human *BRCA1* (sh-BRCA1-B7, sh-BRCA1-G6, and sh-BRCA1-H3). Cells stably expressing sh-Ctrl or shBRCA1 were analyzed by Western blot. B and C, T47D cells stably expressing sh-Ctrl or shBRCA1-G6 (sh-BRCA1) were transplanted into the mammary fat pads of female NSG. Representative gross appearance of tumors formed is shown (B) and tumor volumes were plotted (C). Values represent the average tumor volumes ± SD of four tumors. D–G, mammary tumors formed by transplantation of T47D cells stably expressing sh-Ctrl or sh-BRCA1 were stained with hematoxylin and eosin (H&E; D), TWIST (E), ERα (F), and VIM (G). Note the highly heterogeneous Sh-BRCA1 tumor cells that are positive for TWIST and VIM (black arrows) and negative for TWIST (black arrowheads) and for VIM in Sh-Ctrl. tumors. Large and less-differentiated cells in Sh-BRCA1 tumors are indicated by blue arrows.

expression, expansion of BSCs and CSCs, and induction of EMT and basal-like tumors. These results suggest that either germline mutation of *Brca1* or mammary epithelia-specific deletion of Brca1 is responsible for the activation of EMT-TFs, induction of EMT, dedifferentiation of LSCs, expansion of BSCs and CSCs as well as the development of basal-like tumors. This study provides the first genetic evidence suggesting that Brca1 suppresses EMT and dedifferentiation of LSCs in mammary and tumor development. We also show that KD of BRCA1 in human luminal breast cancer cells activates EMT and increases tumor formation potential, further supporting the data derived from *p18*;*Brca1* double-mutant mice.

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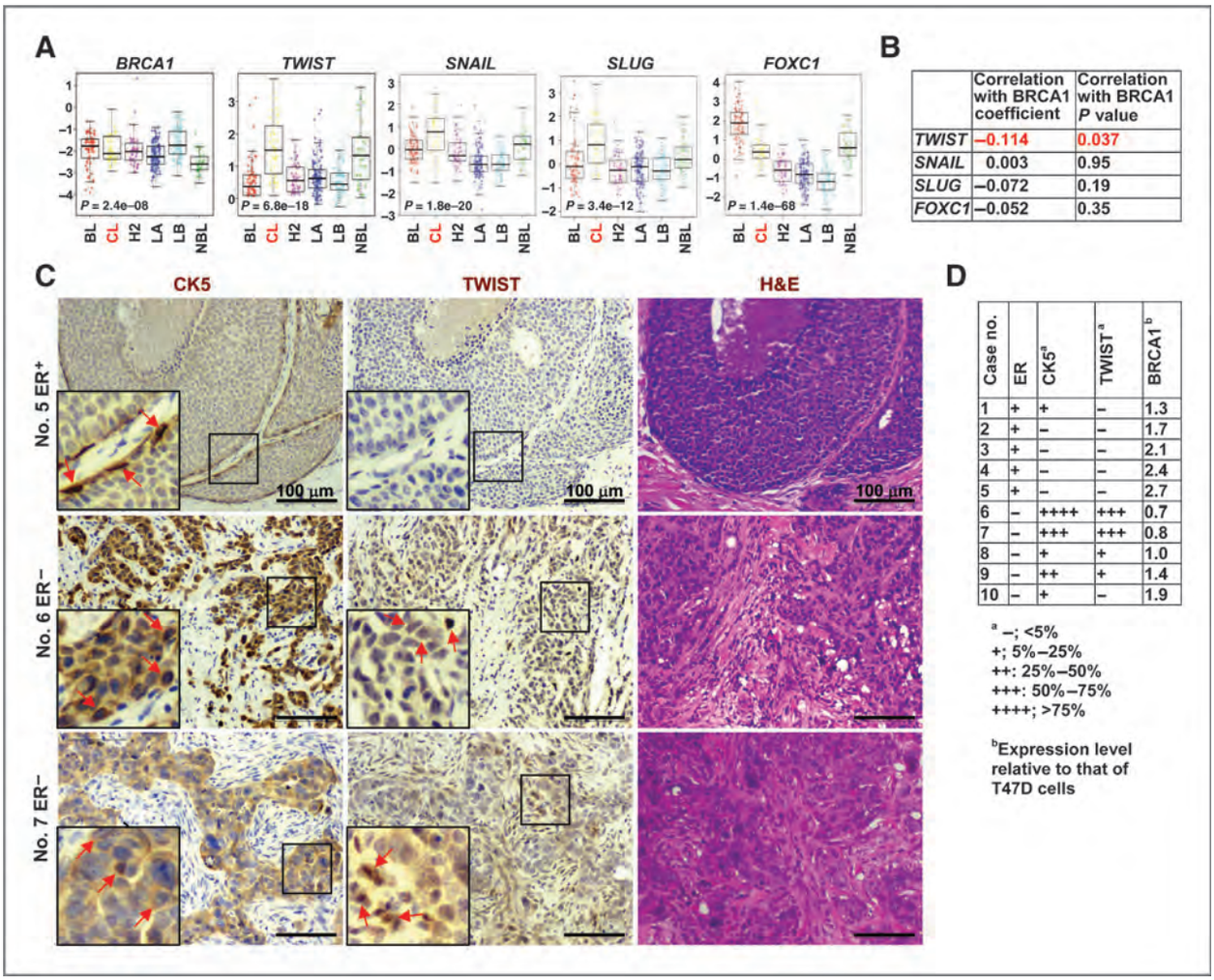

	Correlation with BRCA1 coefficient	Correlation with BRCA1 *P* value
TWIST	−0.114	0.037
SNAIL	0.003	0.95
SLUG	−0.072	0.19
FOXC1	−0.052	0.35

Case no.	ER	CK5[a]	TWIST[a]	BRCA1[b]
1	+	+	−	1.3
2	+	−	−	1.7
3	+	−	−	2.1
4	+	−	−	2.4
5	+	−	−	2.7
6	−	++++	+++	0.7
7	−	+++	+++	0.8
8	−	+	+	1.0
9	−	++	+	1.4
10	−	+	−	1.9

[a] −; <5%
+; 5%–25%
++: 25%–50%
+++: 50%–75%
++++; >75%

[b]Expression level relative to that of T47D cells

Figure 6. BRCA1 and TWIST levels are inversely related to the CL subtype of human breast cancer. A, analysis of gene expression in UNC337 breast cancer patients according to tumor subtype. B, correlation analysis of the expression of *BRCA1* and EMT-TFs for UNC337 breast cancer patients. C, serial sections from ER-positive and -negative invasive human breast cancers were stained with hematoxylin and eosin (H&E), CK5, and TWIST. The boxed areas were enlarged in the insets. Representative cytoplasmic staining of $CK5^+$ cells and nuclear staining of $TWIST^+$ cells are indicated by arrows. D, summary of expression of CK5 and TWIST by IHC and *BRCA1* by qRT-PCR. The expression levels of CK5 and TWIST were scored by the percentage of positive tumor cells in total tumor cells. The expression of *BRCA1* was determined by qRT-PCR. The levels of *BRCA1* mRNA were expressed as the mean values from triplicates of the two sets of the primers.

Because of growth defects induced by Brca1 deficiency (40–42), mice carrying *Brca1* mutation in mammary epithelia rarely develop tumors, making it difficult to identify the cells of origin of *Brca1*-mutant basal-like tumors. Most, if not all, genetic studies have used comutation of one of the genes in the *p53* pathway to overcome the growth defects induced by mutation of *Brca1* in mice (8–9, 40–43). Specific deletion of Brca1 in mammary epithelia by MMTV-Cre or Wap-Cre resulted in basal-like tumor development in p53-deficient mice (40, 43), supporting the notion that the cells of origin of basal-like tumors could be luminal cells. $p53^{f/f}$;$Brca1^{f/f}$;K14-Cre mice targeting deletion in the basal cell lineage developed basal-like tumors, suggesting that *BRCA1*-mutant breast cancer may also arise from basal stem/progenitor cells (44). Direct comparison of $p53^{+/-}$;$Brca1^{f/f}$;K14-Cre and $p53^{+/-}$;$Brca1^{f/f}$;Blg-Cre mice targeting luminal stem/progenitor cells revealed that $p53^{+/-}$;$Brca1^{f/f}$;Blg-Cre tumors phenocopy human *BRCA1*-mutant basal-like breast cancers, whereas $p53^{+/-}$;$Brca1^{f/f}$;K14-Cre tumors do not resemble human *BRCA1* breast cancers, further supporting the notion that *Brca1*-mutant basal-like tumors originate from luminal stem/progenitor cells (9). However, mutation of *p53* in these studies may induce EMT and mammary tumors falling into multiple molecular subtypes, including the basal-like and claudin-low subtypes (45–48), masking the contribution of *Brca1* mutation alone in basal-like tumorigenesis. Hence, it is imperative that the role of Brca1 in controlling mammary stem cells and tumorigenesis be determined under a genetically intact *p53* background.

EMT-TFs orchestrate EMT, which plays an important role in intratumoral heterogeneity and breast basal-like tumor

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progression (49). Recently, Kupperwasser's group reported that *Slug*, an EMT-TF, is aberrantly expressed in the breast of *Brca1*-mutant carriers and that it is necessary for the induction of the basal-like phenotypes of human breast cancers created in mice by transformation of $BRCA1^{mut/+}$ patient–derived breast epithelial cells with four tumorigenic genes (8). Gene-expression profiling of mammary tumors from $p53^{+/-}$;$Brca1^{f/f}$; K14-Cre and $p53^{+/-}$;$Brca1^{f/f}$;Blg-Cre mice showed high mRNA levels of some of the basal and EMT-TFs, including *Foxc1* and *Twist* (9). Furthermore, Brca1 was found to bind to the promoters of *FOXC1* and *FOXC2* and repress their transcription (27). These findings support the function of Brca1 in suppressing EMT-TFs; however, all of these data were derived from either *BRCA1* mutation carriers with unknown patient genetic background, *Brca1*-mutant mice in *p53*-deficient background, or breast cancer cell lines (8, 9, 27). The present study shows, for the first time *in vivo*, that Brca1 suppresses Twist and EMT in LSCs and breast cancer cells in *p53*-intact background.

Deficiency of *Brca1* in mammary stem or tumor cells results in the aberrant expression of almost all EMT-TFs tested (Figs. 1–5). However, only *TWIST* mRNA is significantly, and *FOXC2* mRNA is moderately, suppressed by BRCA1 (Fig. 4). Further supporting these results, our data and previous findings show that BRCA1 binds to putative GATA3 binding sites on the *TWIST* and *FOXC2* promoters, respectively (this report and ref. 27). It remains to be determined whether GATA3 is required for BRCA1 binding and suppression of these genes in mammary stem cells and tumorigenesis *in vivo*. Given the critical roles of TWIST and FOXC2 in regulating EMT and the complex positive regulatory network between these transcription factors (15, 39, 50–53), we believe that Brca1 suppresses EMT, at least partially, by inhibiting TWIST and FOXC2, which, in turn, suppresses other EMT-TFs. Another line of evidence supporting this conclusion is that overexpressing TWIST along with an active form of RAS in mouse mammary luminal cells leads to claudin-low tumors with EMT features and stem cell–like characteristics (54), which molecularly and histologically resembles breast cancers developed in *p18*;*Brca1* double-mutant mice.

Disclosure of Potential Conflicts of Interest

C.M. Perou is a board member for and has ownership interest (including patents) in Bioclassifier LLC and Genecentric Diagnostics. No potential conflicts of interest were disclosed by the other authors.

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References

1. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, et al. Distinct stem cells contribute to mammary gland development and maintenance. Nature 2011;479:189–93.
2. Spike BT, Engle DD, Lin JC, Cheung SK, La J, Wahl GM. A mammary stem cell population identified and characterized in late embryogenesis reveals similarities to human breast cancer. Cell Stem Cell 2012;10:183–97.
3. van Amerongen R, Bowman AN, Nusse R. Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. Cell Stem Cell 2012;11:387–400.
4. Lobo NA, Shimono Y, Qian D, Clarke MF. The biology of cancer stem cells. Annu Rev Cell Dev Biol 2007;23:675–99.
5. Foulkes WD. BRCA1 functions as a breast stem cell regulator. J Med Genet 2004;41:1–5.
6. Liu S, Ginestier C, Charafe-Jauffret E, Foco H, Kleer CG, Merajver SD, et al. BRCA1 regulates human mammary stem/progenitor cell fate. Proc Natl Acad Sci U S A 2008;105:1680–5.
7. Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, et al. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med 2009;15:907–13.
8. Proia TA, Keller PJ, Gupta PB, Klebba I, Jones AD, Sedic M, et al. Genetic predisposition directs breast cancer phenotype by dictating progenitor cell fate. Cell Stem Cell 2011;8:149–63.
9. Molyneux G, Geyer FC, Magnay FA, McCarthy A, Kendrick H, Natrajan R, et al. BRCA1 basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. Cell Stem Cell 2010;7:403–17.
10. Bai F, Smith MD, Chan HL, Pei XH. Germline mutation of Brca1 alters the fate of mammary luminal cells and causes luminal-to-basal mammary tumor transformation. Oncogene 2013;32:2715–25.
11. Wright MH, Calcagno AM, Salcido CD, Carlson MD, Ambudkar SV, Varticovski L. Brca1 breast tumors contain distinct CD44+/CD24− and CD133+ cells with cancer stem cell characteristics. Breast Cancer Res 2008;10:R10.
12. Wicha MS. Cancer stem cell heterogeneity in hereditary breast cancer. Breast Cancer Res 2008;10:105.
13. Wright MH, Robles AI, Herschkowitz JI, Hollingshead MG, Anver MR, Perou CM, et al. Molecular analysis reveals heterogeneity of mouse mammary tumors conditionally mutant for Brca1. Mol Cancer 2008;7:29.
14. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420–8.

Published OnlineFirst September 19, 2014; DOI: 10.1158/0008-5472.CAN-14-1119

15. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 2004;117:927–39.
16. Pei XH, Bai F, Smith MD, Usary J, Fan C, Pai SY, et al. CDK inhibitor p18 (INK4c) is a downstream target of GATA3 and restrains mammary luminal progenitor cell proliferation and tumorigenesis. Cancer Cell 2009;15:389–401.
17. Koboldt DC, Fulton RS, McLellan MD, Schmidt H, Kalicki-Veizer J, McMichael JF, et al. Comprehensive molecular portraits of human breast tumours. Nature 2012;490:61–70.
18. Jonsson G, Staaf J, Vallon-Christersson J, Ringner M, Gruvberger-Saal SK, Saal LH, et al. The retinoblastoma gene undergoes rearrangements in BRCA1-deficient basal-like breast cancer. Cancer Res 2012;72:4028–36.
19. Jiang Z, Deng T, Jones R, Li H, Herschkowitz JI, Liu JC, et al. Rb deletion in mouse mammary progenitors induces luminal-B or basal-like/EMT tumor subtypes depending on p53 status. J Clin Invest 2010;120:3296–309.
20. Cheng L, Zhou Z, Flesken-Nikitin A, Toshkov IA, Wang W, Camps J, et al. Rb inactivation accelerates neoplastic growth and substitutes for recurrent amplification of cIAP1, cIAP2 and Yap1 in sporadic mammary carcinoma associated with p53 deficiency. Oncogene 2010;29:5700–11.
21. Xu X, Wagner KU, Larson D, Weaver Z, Li C, Ried T, et al. Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat Genet 1999;22:37–43.
22. Wagner KU, Wall RJ, St-Onge L, Gruss P, Wynshaw-Boris A, Garrett L, et al. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res 1997;25:4323–30.
23. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, et al. Generation of a functional mammary gland from a single stem cell. Nature 2006;439:84–8.
24. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, et al. Purification and unique properties of mammary epithelial stem cells. Nature 2006;439:993–7.
25. Hollier BG, Tinnirello AA, Werden SJ, Evans KW, Taube JH, Sarkar TR, et al. FOXC2 expression links epithelial-mesenchymal transition and stem cell properties in breast cancer. Cancer Res 2013;73:1981–92.
26. Hill JW, Tansavatdi K, Lockett KL, Allen GO, Takita C, Pollack A, et al. Validation of the cell cycle G(2) delay assay in assessing ionizing radiation sensitivity and breast cancer risk. Cancer Manag Res 2009;1:39–48.
27. Tkocz D, Crawford NT, Buckley NE, Berry FB, Kennedy RD, Gorski JJ, et al. BRCA1 and GATA3 corepress FOXC1 to inhibit the pathogenesis of basal-like breast cancers. Oncogene 2011;31:3667–78.
28. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010;12:R68.
29. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012;486:346–52.
30. Shafee N, Smith CR, Wei S, Kim Y, Mills GB, Hortobagyi GN, et al. Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. Cancer Res 2008;68:3243–50.
31. Buono KD, Robinson GW, Martin C, Shi S, Stanley P, Tanigaki K, et al. The canonical Notch/RBP-J signaling pathway controls the balance of cell lineages in mammary epithelium during pregnancy. Dev Biol 2006;293:565–80.
32. Lim E, Wu D, Pal B, Bouras T, Asselin-Labat ML, Vaillant F, et al. Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. Breast Cancer Res 2010;12:R21.
33. Wagner KU, McAllister K, Ward T, Davis B, Wiseman R, Hennighausen L. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res 2001;10:545–53.
34. Weigelt B, Kreike B, Reis-Filho JS. Metaplastic breast carcinomas are basal-like breast cancers: a genomic profiling analysis. Breast Cancer Res Treat 2009;117:273–80.
35. Keller PJ, Lin AF, Arendt LM, Klebba I, Jones AD, Rudnick JA, et al. Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines. Breast Cancer Res 2010;12:R87.
36. Shakya R, Reid LJ, Reczek CR, Cole F, Egli D, Lin CS, et al. BRCA1 tumor suppression depends on BRCT phosphoprotein binding, but not its E3 ligase activity. Science 2011;334:525–8.
37. Buckley NE, Mullan PB. BRCA1 - conductor of the breast stem cell orchestra: the role of BRCA1 in mammary gland development and identification of cell of origin of BRCA1 mutant breast cancer. Stem Cell Rev 2012;8:982–93.
38. Herschkowitz JI, Zhao W, Zhang M, Usary J, Murrow G, Edwards D, et al. Comparative oncogenomics identifies breast tumors enriched in functional tumor-initiating cells. Proc Natl Acad Sci U S A 2012;109:2778–83.
39. Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci U S A 2010;107:15449–54.
40. Drost RM, Jonkers J. Preclinical mouse models for BRCA1-associated breast cancer. Br J Cancer 2009;101:1651–7.
41. Cao L, Kim S, Xiao C, Wang RH, Coumoul X, Wang X, et al. ATM-Chk2-p53 activation prevents tumorigenesis at an expense of organ homeostasis upon Brca1 deficiency. EMBO J 2006;25:2167–77.
42. Cao L, Li W, Kim S, Brodie SG, Deng CX. Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. Genes Dev 2003;17:201–13.
43. Kumar P, Mukherjee M, Johnson JP, Patel M, Huey B, Albertson DG, et al. Cooperativity of Rb, Brca1, and p53 in malignant breast cancer evolution. PLoS Genet 2012;8:e1003027.
44. Liu X, Holstege H, van der Gulden H, Treur-Mulder M, Zevenhoven J, Velds A, et al. Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. Proc Natl Acad Sci U S A 2007;104:12111–6.
45. Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW, et al. p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat Cell Biol 2011;13:317–23.
46. Wang SP, Wang WL, Chang YL, Wu CT, Chao YC, Kao SH, et al. p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. Nat Cell Biol 2009;11:694–704.
47. Wu WS, Heinrichs S, Xu D, Garrison SP, Zambetti GP, Adams JM, et al. Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma. Cell 2005;123:641–53.
48. Herschkowitz JI, Zhao W, Zhang M, Usary J, Murrow G, Edwards D, et al. Breast Cancer Special Feature: comparative oncogenomics identifies breast tumors enriched in functional tumor-initiating cells. Proc Natl Acad Sci U S A 2011;109:2778–83.
49. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer 2009;9:265–73.
50. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704–15.
51. Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, et al. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. Proc Natl Acad Sci U S A 2007;104:10069–74.
52. Ansieau S, Morel AP, Hinkal G, Bastid J, Puisieux A. TWISTing an embryonic transcription factor into an oncoprotein. Oncogene 2010;29:3173–84.
53. Scheel C, Weinberg RA. Cancer stem cells and epithelial-mesenchymal transition: concepts and molecular links. Semin Cancer Biol 2012;22:396–403.
54. Morel AP, Hinkal GW, Thomas C, Fauvet F, Courtois-Cox S, Wierinckx A, et al. EMT inducers catalyze malignant transformation of mammary epithelial cells and drive tumorigenesis towards claudin-low tumors in transgenic mice. PLoS Genet 2012;8:e1002723.